The human immune response to oral vaccination with live-attenuated *Salmonella* Typhi (Ty21a)

Thesis submitted in accordance with the requirements of

the University of Liverpool for the degree of Doctor of Philosophy

by

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DECLARATION

This thesis is the result of my own work and effort. In some instances, work was completed in conjunction with others. The attribution of work and responsibility is detailed below (Table I). This research was conducted at the Liverpool School of Tropical Medicine and the University of Liverpool. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part, for any other degree or qualification.

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ABSTRACT

Typhoid fever continues to represent a significant threat to global health and currently licensed vaccines confer incomplete protection.

In 1975, an oral typhoid vaccine was developed through the chemical mutagenesis of pathogenic *Salmonella enterica* serovar Typhi strain Ty2. This vaccine, which is designated Ty21a, has been used for many years to combat disease; it is well-tolerated with a cumulative efficacy of approximately 58% up to 2 years following its administration. A great deal of data have been collected concerning peripheral cellular and humoral immune responses to this vaccine; however, only surrogate measures of mucosal immunity exist.

The data presented within this thesis demonstrate the value of direct mucosal sampling and provide fresh insight into aspects of human immunity which have not previously been explored. The data presented describe the strength, diversity and duration of mucosal and peripheral cellular immune responses to Ty21a. In addition, the data demonstrate the wider impact of Ty21a on responses to non-related pathogens. Data presented here support the use of direct mucosal sampling to study host-pathogen interaction and the development of *S.* Typhi based vectors.

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PUBLICATIONS, PRESENTATIONS AND PRIZES

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ABBREVIATIONS

Abbreviations are defined at their first use within the text. The following is a list of abbreviations used within this thesis, compiled in alphabetical order:

APC	Antigen presenting cell
AU	Arbitrary units
B cell	B lymphocyte
BCG vaccine	Bacillus Calmette–Guérin vaccine
BCR	B-cell receptor
BSA	Bovine serum albumin
CCL	Chemotaxis chemokine ligand
CCR	C-C chemokine receptor
CFU	Colony forming unit
CI	Confidence interval
DAPC	Discriminant analysis of principal components
DC	Dendritic cell
EBV	Epstein-Barr virus
EPI	Expanded Programme on Immunisation
FBS	Foetal bovine serum
FOXP3	Forkhead box P3
GCP	Good Clinical Practice
GM-CSF	Granulocyte macrophage colony-stimulating factor
H antigen	Protein flagella
HIV	Human immunodeficiency virus
IFN	Interferon
Ig	Immunoglobulin

ABBREVIATIONS

IL	Interleukin
ILC	Innate lymphoid cell
iMFI	Integrated mean fluorescence intensity
LPS	Lipopolysaccharide
M cell	Microfold-cell
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MAIT cell	Mucosal-associated invariant T cell
MDR	Multidrug resistant
MFI	Geometric mean fluorescence intensity
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
miRNA	MicroRNA
MMC	Mucosal mononuclear cell
NHS	National Health Service
NK cell	Natural killer cell
NKT cell	Natural killer T cell
NRES	United Kingdom National Research Ethics Service
OMPs	Outer membrane proteins
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCV	13-valent pneumococcal conjugate vaccine
PPD	Purified protein derivative
REC	Research Ethics Committee
S. Typhi	*Salmonella enterica* subspecies I serovar Typhi
S. Typhimurium	*Salmonella enterica* serovar Typhimurium

ABBREVIATIONS

SCV	*Salmonella* containing vacuole
SEB	Staphylococcal enterotoxin B
SPI	*Salmonella* Pathogeneicity Island
T cell	T lymphocyte
T3SS	Type III secretion system
T_{CM}-cell	Central memory T-cell
TCR	T-cell receptor
T_{EM}-cell	Effector memory T-cell
T_{EMRA}-cell	Terminal effector memory T-cell
TGF	Transforming growth factor
T_H cell	Helper $CD4^+$ T cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_{REG} cell	Regulatory T cell
Vi polysaccharide	Virulence polysaccharide
WHO	World Health Organisation

CHAPTER 1: INTRODUCTION

Salmonella enterica subspecies I serovar Typhi (*S*. Typhi) is a facultative intracellular pathogen and the causative agent of typhoid fever. This bacterium is highly adapted to its human host and possesses a number of specialised mechanisms, which combine to facilitate survival, replication and transmission. Disease is endemic in many resource-poor nations, where inadequate sanitation and poor food hygiene facilitates faecal-oral transmission.

For a long time, vaccination has been used to combat disease. Unfortunately, currently licensed vaccines confer incomplete protection and are ineffective or unsuitable for use in young children. Following the successful introduction of antibiotic treatment, the pressure to develop new vaccines was reduced and interest waned. The emergence of antimicrobial resistant strains has redirected efforts towards the development of new vaccines. Unfortunately, development has been hindered by our limited understanding of mucosal immune defence, mechanisms of protection and the absence of functional correlates of protection.

1.1. The species

S. enterica is a facultative intracellular Gram-negative motile bacillus which comprises 6 subspecies, 50 serogroups and more than 2,600 serovars (Issenhuth-Jeanjean et al., 2014).

Approximately 99% of serovars which cause illness in humans reside within subspecies I (Desai et al., 2013). The vast majority of these serovars have a broad host rage and cause self-limiting gastroenteritis in humans. These non-typhoidal serovars actively induce inflammation at the intestinal lumen and outcompete other bacteria through the extraction of inflammatory-derived nutrients (Winter et al., 2010). In the presence of immunosuppressive illness, non-typhoidal serovars can cause an invasive bloodstream infection in humans, known as invasive non-typhoidal disease (Feasey et al., 2015).

A small number of serovars are human-host-restricted and cause typhoid/paratyphoid. These typhoidal serovars are believed to have evolved along four phylogenetically unrelated lineages; *S*. Typhi, *S*. Paratyphi B and *S*. Paratyphi C each form single lineages, while the fourth lineage is formed by *S*. Paratyphi A and *S*. Sendai (Selander et al., 1990). It is likely that typhoidal serovars are descended from organisms that once caused gastroenteritis in humans. It has been demonstrated that, while 204 genes are functionally disrupted or inactive in the typhoidal serovar *S*. Typhi, more than 80% their homologs are functional in the non-typhoidal serovar *S*. Typhimurium (McClelland et al., 2001).

1.2. The serovar

S. Typhi is believed to have emerged between 10,000 and 71,000 years ago (Roumagnac et al., 2006) and was first identified as the causative agent of typhoid by Karl Joseph Eberth in 1880 (Marineli et al., 2013). Due to the risk of human infection, *S.* Typhi is classified as a containment level 3 organism (Kohler, 2005).

The Kauffman–White scheme utilises antisera to classify serovars according to the expression of lipopolysaccharide (LPS) O-antigens, flagella H-antigens and the virulence (Vi) polysaccharide antigen (Judicial Commission of the International Committee on Systematics of, 2005). *S.* Typhi is placed in group D based on the expression of LPS O-9 and O-12 antigens, the flagella H1-d antigen and the Vi polysaccharide antigen (Figure 1.1). When cultured on blood agar, the bacteria form smooth white colonies, approximately 2 to 3 mm in diameter (Figure 1.2).

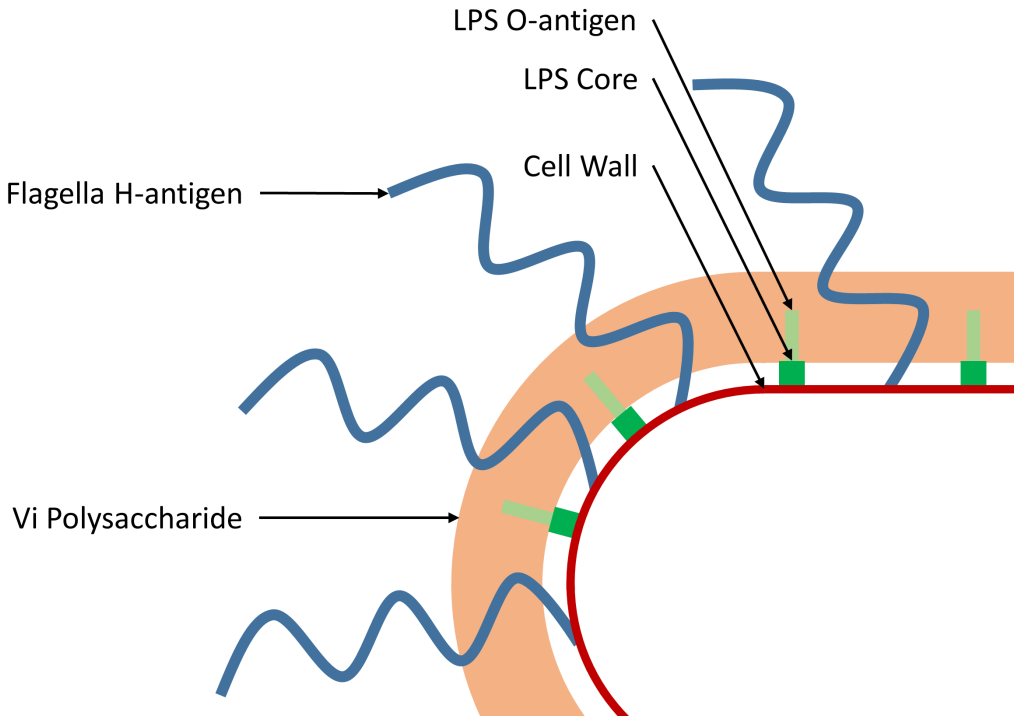

Figure 1.1. A diagrammatic representation of a section of *Salmonella* Typhi showing the principal antigens

Key structures are shown, including the cell wall, lipopolysaccharide (LPS) core and O-antigen, the virulence (Vi) polysaccharide and flagella H-antigen.

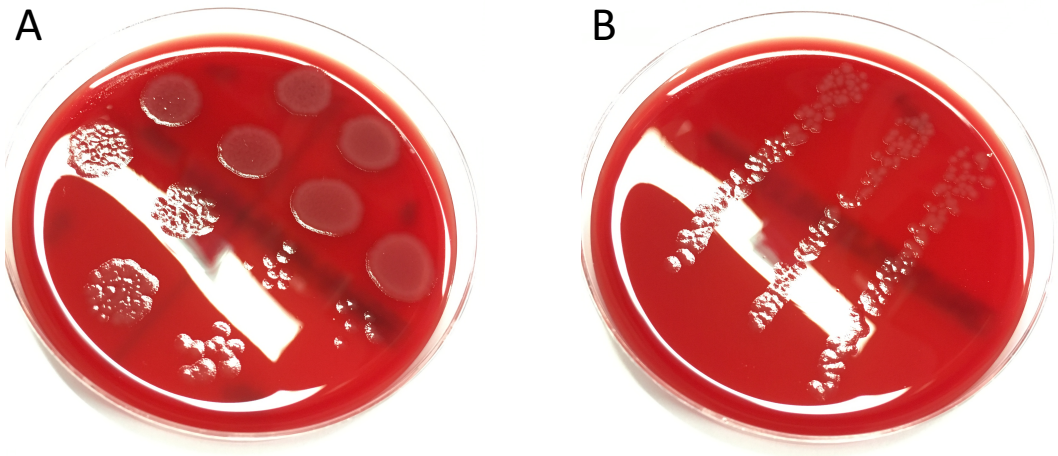

Figure 1.2. *Salmonella* Typhi strain Ty21a grown on Columbia blood agar

(A) Four dilutions plated as three 10 µL spots. (B) A single dilution plated as three 10 µL streaks.

There is approximately 1% variation in the core genome of serovars within *S. enterica* (Baker and Dougan, 2007). Alongside the core genome, there are virulence associated regions termed *Salmonella* Pathogenicity Islands (SPIs), which are thought to have been acquired relatively recently through horizontal gene transfer (Hacker and Kaper, 2000). SPI-1 and SPI-2 encode type III secretion systems (T3SSs), these needle-like structures facilitate manipulation of host-cell function through effector protein secretion (Figure 1.3) (Sukhan, 2000). SPI-7 encodes the capsular Vi polysaccharide, which masks bacterial antigens (Nair et al., 2004). The vast majority of *S*. Typhi isolates that have been sequenced possess SPI-7 (Wain et al., 2005), which suggests that expression of the Vi polysaccharide provides a substantive selective advantage. Indeed, it has been demonstrated that strains which express the Vi polysaccharide are more virulent than those that do not (Hornick et al., 1970, Hone et al., 1988).

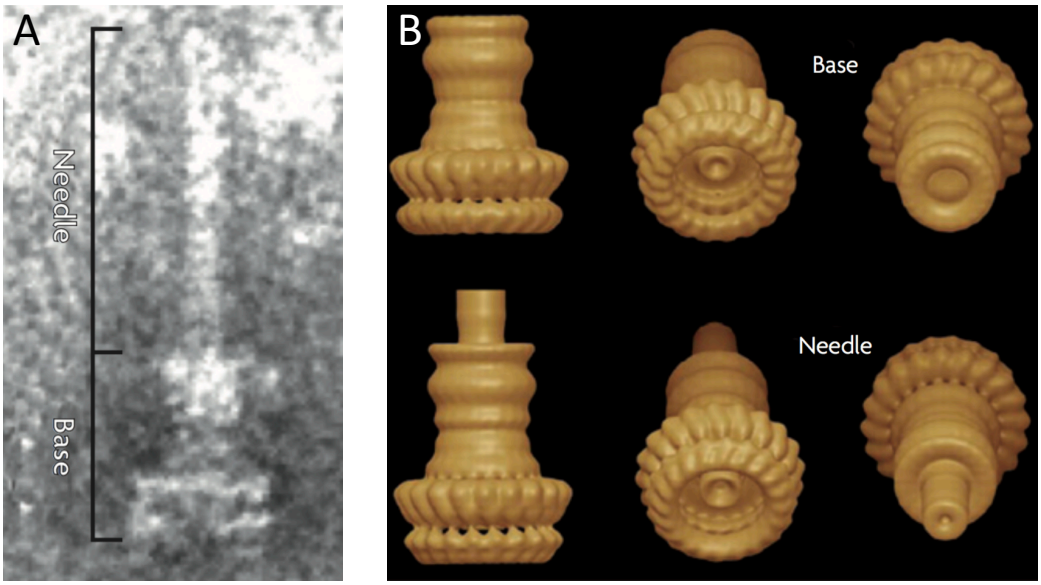

Figure 1.3. Structure of the *Salmonella* type III secretion systems (T3SS) (Haraga et al., 2008)

(A) Electron micrographs of a *Salmonella enterica* serovar Typhimurium T3SS. (B) Electron cryomicroscopy and surface images of the structures of a S. Typhimurium T3SS.

S. Typhi typically possesses a single, monophasic flagella encoded on the *FliC* locus (Frankel et al., 1989). Flagella are involved in motility and cell invasion (Grossman et al., 1995). *S.* Typhi typically expresses the H1-d antigen; however, 16% of Indonesian isolates alternately express the H1-j antigen (Grossman et al., 1995). Evidence suggests that isolates which express the H1-j antigen are less motile and less invasive than those which express the H1-d antigen (Grossman et al., 1995). Interestingly, however, H1-j expression is more common among isolates collected from the elderly (Grossman et al., 1995). It is possible that expression of H1-j confers a selective advantage in the elderly through the evasion of adaptive immune responses generated through repeated environmental exposure to H1-d.

Expression of these bacterial components varies according to the stage of the infection. High osmolarity in the intestinal lumen is thought to inhibit Vi polysaccharide expression, which facilitates bacterial adhesion and invasion across the intestinal mucosa (Raffatellu et al., 2007). Within the mucosa, low osmolarity leads to expression of *tviA*, which results in

expression of the Vi polysaccharide and inhibition of SPI-1 and flagella expression (Raffatellu et al., 2007). Expression of the Vi polysaccharide prevents complement activation via the O-antigen (Wilson et al., 2011) and the ligation of host-cell toll-like receptors (TLRs) via pathogen associated molecular patterns (PAMPs) (Raffatellu et al., 2005, Wilson et al., 2008). Expression of the Vi polysaccharide has also been shown to inhibit bacterial-guided neutrophil chemotaxis (Wangdi et al., 2014). Recognition of flagellin, the major subunit of flagellum, within a cells cytoplasm results in inflammasome activation and cell death (Winter et al., 2015). Thus, inhibition of flagella expression within the intracellular space facilitates intracellular survival.

1.3. Pathogenesis

S. Typhi is restricted to its human host and the bulk of our understanding regarding disease pathogenesis has been gathered from murine models of *S*. Typhimurium infection and models utilising human cell-lines.

Following ingestion, prior to invasion, sufficient numbers of bacteria must weather gastric acidity and avoid enzymatic degradation. Individuals with impaired hydrochloric acid production, either as a result of antacid consumption or gastrectomy, are at increased risk of developing disease (Parry et al., 2002). SPI-1 plays a prominent role in the establishment of infection; through effector protein secretion, it disrupts the epithelial barrier and facilitates host cell invasion through membrane and cytoskeletal rearrangement (Scherer et al., 2000, Hayward et al., 2000, Zhou et al., 2001). *S*. Typhi preferentially invades via microfold (M)-cells overlying Peyer's patches in the terminal ileum (Kohbata et al., 1986, Jepson and Clark, 2001). M-cells are specialised epithelial cells which are involved in sampling antigen from the intestinal lumen. Due to their folded basal structure, M-cells are able to associate closely and deliver antigen to underlying immune cells through transcytosis (Jepson and Clark, 2001). *S.* Typhi may also invade via intestinal epithelial cells (Takeuchi and Sprinz,

1967), or through transmigration following disruption of the epithelial barrier (Kops et al., 1996).

Once the intestinal barrier has been breached, salmonellae invade immune cells, primarily macrophage cells, where they persist within *Salmonella* containing vacuoles (SCVs) (Alpuche-Aranda et al., 1994, Richter-Dahlfors et al., 1997). SPI-2 plays an important role in intracellular survival within SCVs; through effector protein secretion, it manipulates host-cell function in order to facilitate bacterial replication and dissemination (Figure 1.4) (Haraga et al., 2008). Murine models have demonstrated that deletion of TLR-2, TLR-4 and TLR-9 inhibits upregulation of SPI-2 and reduces bacterial virulence (Arpaia et al., 2011). These data indicate that the ligation of multiple TLRs is required for virulence *in vivo*. Salmonellae typically infect a high proportion of cells at low levels (Brown et al., 2006). Intracellular populations are typically heterogeneous, comprising both replicating and non-replicating bacteria (Helaine et al., 2014). Mathematical models predict that *S.* Typhi may be released from the intracellular environment into the extracellular space at any time, independent of the intracellular burden (Brown et al., 2006, Dybowski et al., 2015). These phenomena combine to facilitate efficient systemic bacterial dissemination.

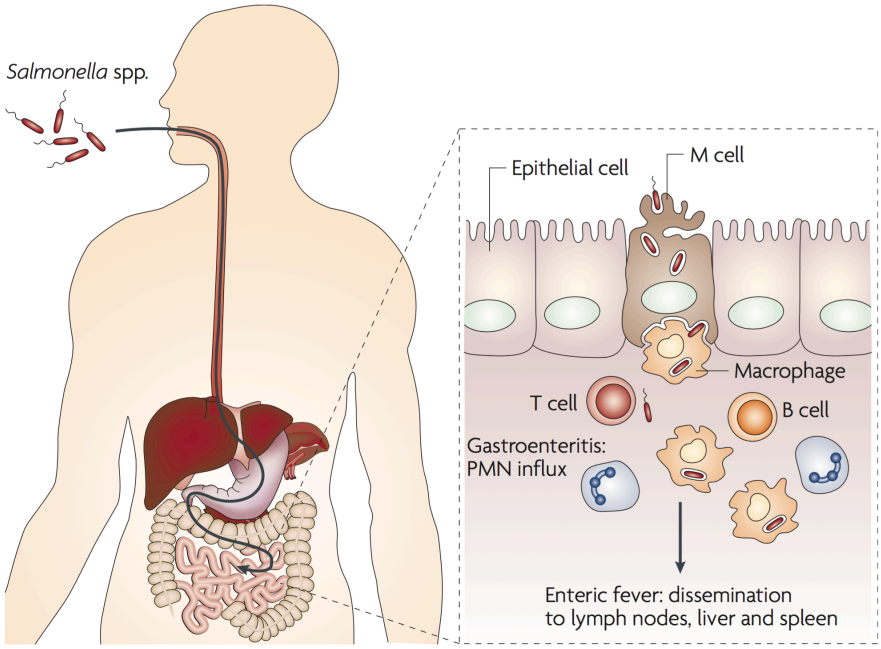

Figure 1.4. Invasion of *Salmonella* via microfold (M)-cells (Haraga et al., 2008)

Intracellular salmonellae may disseminate via lymphatics or blood throughout the reticuloendothelial system (Parry et al., 2002). Extracellular dissemination may also occur; however, within the extracellular compartment, the bacteria are susceptible to B-cell-mediated mechanisms of defence (Cunningham et al., 2007). Dissemination facilitates evasion of the progressive local inflammatory response and ensures that bacterial replication does not become nutritionally or spatially limited (Mastroeni and Grant, 2011).

Data acquired from bacteraemic patients demonstrate that the bacterial burden in blood is higher in children than in adults (Saha et al., 2001). It is possible that, in adults, established adaptive immune responses, generated through repeated environmental exposure, limit bacterial replication. *S.* Typhi in peripheral circulation are trapped by Kuppfer cells in the liver and are subsequently secreted in bile leading to reinfection of Peyer's patches, reinvasion via the small bowel and shedding in stool (Nguyen et al., 2004).

1.4. Global burden of disease

The burden of typhoid fever is greatest among the resource-poor nations of Asia and sub-Saharan Africa (Crump and Mintz, 2010). In resource-rich nations, cases are largely restricted to travellers returning from regions of endemic disease (Mermin et al., 1998, Ackers et al., 2000).

Isolation of *S*. Typhi from bone marrow is currently the gold standard diagnostic for typhoid fever and isolation from blood the next best (Wain and Hosoglu, 2008). In the developing world, where the burden of disease is greatest, bacterial culture is not routinely performed and there remains a dependence upon other less-accurate methods. The Widal test, which is based on the detection of serum immunoglobulin to O-antigens and H-antigens, is the most commonly used method to inform diagnoses in the developing world. Unfortunately, geographical baseline variation, immunoglobulin cross-reactivity and non-specific seroconversion, can result in the generation of misleading diagnostic output (Olopoenia and King, 2000).

In 2010, the World Health Organisation (WHO) estimated that there are approximately 26.9 million cases of disease annually, with an associated mortality of 1% (Buckle et al., 2012). More recently, meta-regression has estimated that there are approximately 17.8 million cases of disease in low-income and middle-income countries (Antillon et al., 2017). Unfortunately, since these estimates remain dependent on the extrapolation of unreliable data sets, they are unlikely to reflect the true burden of disease (Crump et al., 2004, Mweu and English, 2008, Ochiai et al., 2008, Breiman et al., 2012).

1.5. Age related burden of disease

It is estimated that 58% of all cases of disease in endemic regions occur in children under 5 years (Buckle et al., 2012). A study conducted in Delhi demonstrated that the incidence of disease was 27.3 per 1,000 person years in children less than 5 years, 11.7 in those aged

between 5 and 19 years and 1.1 in those aged between 19 and 40 years (Sinha et al., 1999). A study conducted in Dhaka demonstrated that the incidence of disease was 18.7 per 1,000 person years in children aged less than 5 years and 2.1 in those aged more than 5 years (Brooks et al., 2005). Interestingly, variation has been observed in children aged less then 5 years, with 4% being diagnosed with typhoid in the first year of life compared with 85% in children aged between 2 and 4 years (Brooks et al., 2005). This may be due to a reduced level of exposure to *S*. Typhi during early life, or as a result of protection conferred through the passive transfer of maternal immunoglobulin.

1.6. Controlled human infection

Data collected from murine models of *S*. Typhimurium infection are of limited value. A murine model of *S.* Typhi infection has been established through deletion of TLR-11 (Mathur et al., 2012); however, the reproducibility of this model is variable (Song et al., 2016, Mathur et al., 2016). Humanised murine models have also been developed, through engraftment of severe combined immunodeficiency mice with human hematopoietic stem cells; however, these models are costly and subject to variability as a result of the genetic heterogeneity of donors and the variable degree of engraftment (Firoz Mian et al., 2011).

Considerable insight has been gleaned from studies of natural *S*. Typhi infection in endemic regions; however, through controlled human infection it is possible to study transmission, pathogenesis, immunity and prophylactic intervention, in a setting where baseline measurements may be made and the exact time of bacterial challenge is known (Pollard et al., 2012). However, it should be noted that there are considerable differences between populations enrolled in controlled human infection and those at risk in endemic regions, both with regards age and the level of natural exposure to the pathogen. Despite this, through controlled human infection it may be possible to identify functional correlates of protection, which would help facilitate the development and licensure of new vaccines.

In 1962, the first model of controlled human infection with *S.* Typhi was established at the University of Maryland (Hornick et al., 2007). In this model, participants were orally challenged with virulent *S.* Typhi strain Ty2 suspended in milk. Two different strains were used in this model; early trials used a strain which was isolated in Kherson (modern day Ukraine) in 1918 (Hornick et al., 2007) and latter trials used a strain which was isolated from the gallbladder of a known chronic carrier in 1958 (Quailes strain) (Darton et al., 2014). Controlled human infection at the University of Maryland was instrumental in the validation of chloramphenicol treatment and for the licensure of the live-attenuated oral Ty21a vaccine (Hornick et al., 2007). In 1974, due to ethical issues surrounding the use of prison inmates as study participants, this model was terminated (Lerner, 2007).

More recently, in 2014, a model of controlled human infection with *S.* Typhi was established at the University of Oxford (Waddington et al., 2014). In this model, volunteers are orally challenged with the same Quailes strain originally used at the University of Maryland. In this model, 10^3 colony forming units (CFUs) suspended in a sodium bicarbonate solution has an attack rate of 55% and 10^4 CFUs an attack rate of 65% (Waddington et al., 2014). Already, a number of licensed and next-generation vaccines have been trialled (Darton et al., 2016, Jin et al., 2017) and several novel findings concerning host-pathogen interaction have been reported (Toapanta et al., 2015, McArthur et al., 2015, Toapanta et al., 2016, Blohmke et al., 2016, Fresnay et al., 2016, Darton et al., 2017). Data generated using this model are discussed in greater detail in subsequent sections of this thesis.

1.7. Clinical presentation and treatment

In healthy volunteers, the typical infectious dose of *S*. Typhi ranges between 10^3 and 10^9 CFUs (Hornick et al., 1970) and the time from ingestion to clinical presentation ranges between 3 and 21 days (Gordon, 2008). Typhoid fever produces a wide range of symptoms and most commonly presents as a non-specific febrile illness with hepatosplenomegaly and abdominal

pain (Gordon, 2008). In some instances these symptoms may be accompanied by chills, nausea, anorexia, diarrhoea, constipation, headache and bradycardia (Gordon, 2008).

Introduced in 1948, chloramphenicol was the first antibiotic used to treat patients diagnosed with typhoid (Woodward et al., 1948). Chloramphenicol reduced case fatality from 26% in 1948 to 5% in 1990 (van den Bergh et al., 1999). Treatment also reduced recovery time as well as the risk of secondary complications (van den Bergh et al., 1999). In 1950, just 2 years after the introduction of chloramphenicol, the first chloramphenicol-resistant isolates were identified (Colquhoun and Weetch, 1950). By 1975, more than 80% of all isolates collected from Vietnam were resistant to chloramphenicol (Linh and Arnold, 1975). Initially, these isolates retained sensitivity to co-trimoxazole, ampicillin and amoxicillin; however, in the 1980s, the first multidrug resistance (MDR) isolates, resistant to all frontline antibiotics, were identified (Bhutta et al., 1991).

Available data indicate that the prevalence of MDR is geographically variable, with MDR observed in 0% of Chinese isolates, 0% of Indonesian isolates, 7% of Indian isolates, 22% of Vietnamese isolates, 65% of Pakistani isolates (Ochiai et al., 2008). More recently, during a recent epidemic in Blantyre in 2014, 97% of all isolates were identified as MDR (Feasey et al., 2015). This epidemic was associated with the emergence of the H58 lineage. H58 has spread throughout Africa and Asia over the last 30 years, displacing antibiotic-sensitive isolates and reshaping the global population structure of this pathogen (Wong et al., 2015).

Fluoroquinolone antibiotics, namely ciprofloxacin and ofloxacin, are recommended for the treatment of infection with MDR *S*. Typhi (Dutta et al., 1993). These antibiotics are ideally suited for the treatment of typhoid fever since they are concentrated in macrophages, bile and the bowel, they are effective within the intracellular compartment and have an oral efficacy equivalent to that achieved through intravenous administration (Hooper and Wolfson, 1991). The efficacy of these drugs for the treatment of infection with MDR *S*. Typhi

in all age groups has been established in randomised control trials (Cao et al., 1999, Girgis et al., 1999). Data indicate that fluoroquinolones have 97.9% clinical efficacy, typically resolving fever in just 3.9 days (Parry et al., 2002).

Fluoroquinolones are not licensed for use in children due to concern that these drugs could cause joint toxicity (Burkhardt et al., 1990). Off-license use of these drugs, where the risk has been deemed clinically acceptable, has provided evidence supporting the safety of fluoroquinolones in children (Schaad et al., 1991, Schaad and Wedgwood, 1992). In one study, children aged between 1 and 14 years treated with fluoroquinolones presented with no evidence of join toxicity and had comparable growth compared with age-matched controls (Bethell et al., 1996).

In 1992, the first fluoroquinolone-resistant isolates of *S*. Typhi were identified (Umasankar et al., 1992). The prevalence of these strains has increased, rising from 4% in 1993 to 76% in 1998 in Vietnam (Parry et al., 1998) and from 2% in 1998 to 40% in 2001 in India (Mehta et al., 2001). While isolates of *S*. Typhi have been identified which are fully resistant to ciprofloxacin, they have, thus far, retained susceptibility to ceftriaxone and azithromycin (Chuang et al., 2009). Ceftriaxone is as effective as chloramphenicol over a 3 day or 7 day course (Lasserre et al., 1991, Islam et al., 1988). Treatment of fluoroquinolone-resistant isolates is dependent upon the delivery of high dosage therapy and is associated with a prolonged period of infection and an increased risk of treatment failure (Wain et al., 1997, Parry et al., 1998).

1.8. Innate immunity

Immunity to typhoid is complex and our understanding of the mechanisms which mediate protection incomplete. Cells of the innate immune system are able to efficiently engage pathogens immediately, in a semi-specific manor, via TLRs which recognise PAMPs. Innate

immune cells play a prominent role in early defence as well as in the induction of adaptive immune responses.

Dendritic cells (DCs) are professional antigen presenting cells (APCs), derived from monocytes, which are believed to play a particularly important role in the generation of T-cell-mediated immunity to typhoid. Infected DCs are able to present *S*. Typhi antigens directly sampled from within their cytoplasm (Salerno-Goncalves and Sztein, 2009). DCs are also able to phagocytose *S*. Typhi infected cells and present antigens through suicide cross-presentation (Salerno-Goncalves and Sztein, 2009). DCs may also activate T cells through cytokine production (Salerno-Goncalves and Sztein, 2009, Toapanta et al., 2015).

Controlled human infection has demonstrated that, in volunteers resistant to infection, monocyte and DC capacity for binding is increased immediately following challenge (Toapanta et al., 2015). In contrast, in volunteers susceptible to infection, monocyte and DC capacity for binding is only increased during the course of infection, in the days following diagnosis of disease (Toapanta et al., 2015). This suggests that monocytes and DCs play an important role in early defence and also likely contribute towards the elimination established infection.

Murine models have demonstrated that epithelial TLR-5 ligation by flagella induces expression of chemotaxis chemokine ligand (CCL)20 which results in the migration of immature DCs to the intestinal mucosa (Sierro et al., 2001). Controlled human infection has demonstrated that, in volunteers susceptible to infection, in the days leading to and immediately following diagnosis of disease, $\alpha_4\beta_7$ is upregulated among monocytes but not among DCs (Toapanta et al., 2015). No changes were observed in volunteers resistant to infection among either monocytes or DCs (Toapanta et al., 2015). This suggest that, in humans, monocytes and DCs migrate to different tissues during the course of infection.

Murine models of infection have observed the accumulation of neutrophils in Peyer's patches and mesenteric lymph nodes during the course of infection (Rydstrom and Wick, 2007). Neutrophils are a prominent source of interferon (IFN)-γ during acute infection and their depletion is associated with increased bacterial burden in the liver 24 hours following challenge (Conlan, 1996, Spees et al., 2014). In humans and mice, interleukin (IL)-12 or IFN-γ deficiency is associated with increased susceptibility to Infection (Jouanguy et al., 1999). These data suggest that neutrophils contribute toward early defence through the inhibition of bacterial dissemination.

1.8.1. Mucosal homing

The mucosal surface of the gastrointestinal tract is protected by specialised components of the innate and adaptive immune system (Figure 1.6). These cell populations facilitate the generation of specialised immune responses tailored to defence against mucosal pathogens.

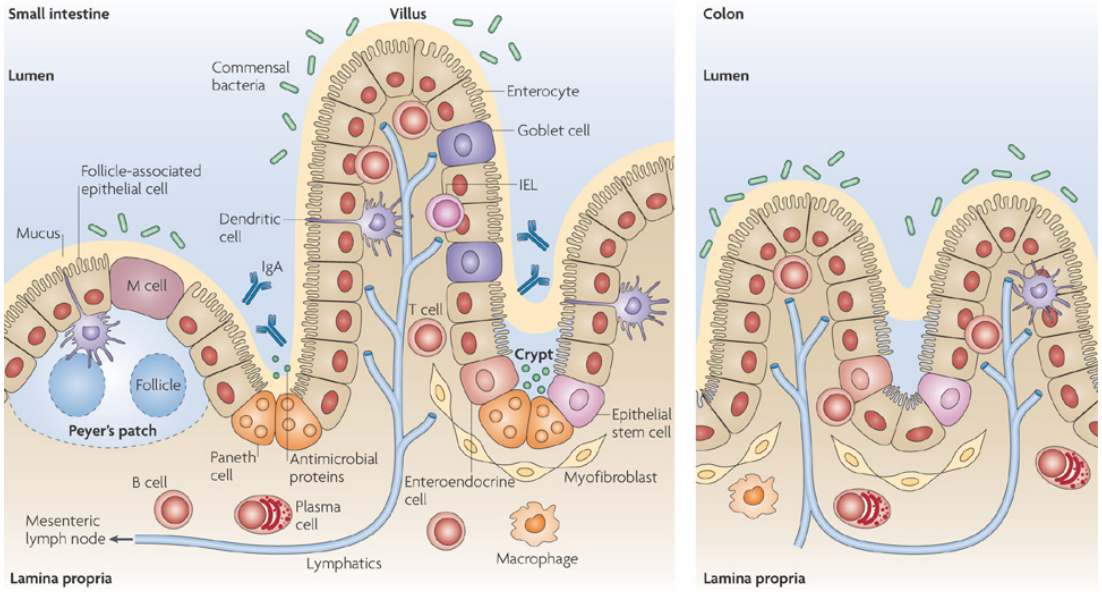

Figure 1.6. Defences of the gastrointestinal tract (Abreu, 2010)

M-cells, which overly Peyer's patches in the small intestine, are responsible for sampling antigen from the intestinal lumen. Their complex folded basal structure facilitates close association with innate and adaptive immune cell subsets. DCs found at the intestinal

mucosa possess a unique morphology which facilitates antigen sampling from within the intestinal lumen (Soloff and Barratt-Boyes, 2010). Gut-associated DCs, unlike extra-intestinal DCs, are able to metabolise vitamin A in order to produce all-trans retinoic acid, which induces expression of $\alpha_4\beta_7$ and C-C chemokine receptor (CCR)9 on adaptive immune cells (De Calisto et al., 2012). Migration of adaptive immune cells is dependent upon interactions between these molecules and others expressed at the intestinal mucosa; specifically, $\alpha_4\beta_7$ interacts with mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and CCR9 interacts with CCL25 (De Calisto et al., 2012, Mavigner et al., 2012). Cellular residence at the intestinal mucosa is, in part, mediated through the expression of $\alpha_\varepsilon\beta_7$ (Berlin et al., 1993, Cepek et al., 1994).

Through oral vaccination it is possible to utilise these specialised mechanisms of defence to more efficiently target pathogens which invade via the intestinal mucosa. As a result, during the assessment of oral vaccines, the expression of $\alpha_4\beta_7$ and CCR9 is commonly assessed in peripheral blood as a surrogate measure of mucosal immunogenicity (Pasetti et al., 2011).

1.9. Adaptive immunity

Data collected at a Royal Air Force base in Egypt, during two separate outbreaks of disease, revealed an attack rate of 20.4% in individuals previously diagnosed with typhoid, compared with 31.2% in individuals not previously diagnosed with typhoid (Marmion et al., 1953). Similarly, controlled human infection has demonstrated an attack rate of 23% in volunteers previously diagnosed with typhoid, compared with 30% in naïve volunteers not previously diagnosed with typhoid (Dupont et al., 1971). These data suggest that wild-type *S*. Typhi is able to evade adaptive immune responses generated during the course of infection; however, since currently licensed vaccines do confer a moderate level of protection, it is clear that the human immune system, if correctly primed, has the potential to defend against disease.

The adaptive immune system serves as the basis for vaccine-mediated prophylaxis (Levine and Sztein, 2004). Broadly speaking, adaptive immunity is conferred through two lymphocyte populations – B lymphocytes (B cells) and T lymphocytes (T cells). These populations possess unique effector functions and both likely contribute towards immunity to typhoid. The Vi polysaccharide vaccine is effective, which demonstrates that B cells alone can confer protection against disease; however, since intracellular bacteria are largely protected from immunoglobulin effector function, T cells likely play a prominent role in the elimination of established intracellular infection.

1.10. B-cell-mediated immunity to *S*. Typhi

B cells are able to recognise antigens secreted by a pathogen as well as those expressed on their surface. Recognition of antigen by the B-cell receptor (BCR) expressed on the surface of B cells results in activation of effector function. In a T-cell-dependent fashion, memory B cells may be generated.

During extracellular phases of the infection cycle, *S*. Typhi are susceptible to B-cell-mediated mechanisms of defence. The importance of intracellular immune evasion has been demonstrated by murine models which have demonstrated that salmonellae which are unable to establish themselves within the intracellular niche are avirulent (Fields et al., 1986).

1.10.1. Memory B cells to *S*. Typhi

Memory B cells are long-lived cells which are responsible for preservation of immunity to the antigen recognised by the BCR. Following oral vaccination with live-attenuated *S*. Typhi, IgG and IgA memory B cells specific to flagella, Vi polysaccharide and LPS have been observed in peripheral blood for up to 1 year (Wahid et al., 2011). Controlled human infection has demonstrated that in volunteers susceptible to infection, around the time of diagnosis, fewer memory B cells persist in peripheral blood and there is a reduction in the expression of $\alpha_4\beta_7$

(Toapanta et al., 2016). Based on these data, it is reasonable to hypothesise that memory B cells expressing high levels of $\alpha_4\beta_7$ are depleted from peripheral blood and sequestered at the intestinal mucosa.

1.10.2. Plasma B cells to *S*. Typhi

Memory B cells, following recognition of antigen via the BCR, are able to rapidly differentiate into plasma B cells. These cells secrete large quantities of immunoglobulin specific to the antigen recognised by the BCR. Factors which have been shown to influence the strength of plasma B-cell responses in humans include:

a) Vaccine type – live vaccines produce stronger plasma B-cell responses compared with inactivated vaccines (Kantele et al., 1991).

b) Vaccine dosing schedule – three doses of Ty21a produces stronger plasma B-cell responses than two doses. Six doses, does not impact the strength of the of plasma B-cell responses, but does extended the period of peripheral circulation (Kantele, 1996).

c) Vaccine formulation – whether vaccines are administered via gelatine capsules, enterically-coated capsules or as a liquid suspension influences the strength of plasma B-cell responses (Kantele, 1990, Kantele, 1996). In models of controlled human infection, stomach acid neutralisation facilitates the use of lower dose inoculum and results in a more consistent pattern of clinical infection (Waddington et al., 2014).

Following oral vaccination with attenuated *S*. Typhi, IgA plasma B cells are most abundant, IgM plasma B cells are the second most abundant, with only low numbers of IgG plasma B cells observed in peripheral blood; however, these observations are subject to considerable inter-individual variation (Kantele, 1990, Kantele, 1996). It has previously been

demonstrated that levels of serum immunoglobulin correlate with plasma B-cell numbers; however, when plasma B-cell numbers are low, levels of immunoglobulin in sera may drop below the lower limit of detection (Kantele, 1990). Plasma B cells are most abundant between 4 and 10 days post-vaccination (Kantele et al., 1986, Kantele, 1990, Kantele et al., 1991). Following a period of peripheral circulation, plasma B cells are believed to migrate to mucosal effector sites; however, in cases of prolonged exposure to antigen, extended periods peripheral circulation have been observed (Kantele, 1996).

1.10.1. Mucosal homing of plasma B cells to *S*. Typhi

Controlled human infection has demonstrated that that in volunteers susceptible to disease, around the time of diagnosis, plasma B cells in peripheral blood express higher levels of $\alpha_4\beta_7$ (Toapanta et al., 2016). Through high level expression $\alpha_4\beta_7$, these plasma B cells may rapidly migrate to the intestinal mucosa and contribute towards clearance of the pathogen.

Plasma B cells generated through oral vaccination with attenuated *S*. Typhi have the same receptor profile as those generated through natural infection (Kantele et al., 2013). The majority of IgA plasma B cells, primed through oral vaccination, express the mucosal homing receptor $\alpha_4\beta_7$ (Kantele et al., 1997, Kantele et al., 1999a). IgG and IgA $\alpha_4\beta_7^+$ plasma B cells specific to LPS have been observed which do and do not express CD62L. This is consistent with populations having the capacity to migrate to intestinal and peripheral lymphoid tissue (Wahid et al., 2012). It is likely that these populations contribute towards the elimination of intestinal and systemic infection, respectively.

1.10.2. Immunoglobulins to *S*. Typhi

Immunoglobulins are potent immune effector molecules. They are comprised of a variable region, which facilitates antigen recognition and a constant region, which defines function; IgG has two high-affinity antigen binding domains and accounts for approximately 75% of all

serum immunoglobulin; IgA has four high-affinity antigen binding domains and is most abundant in mucosal secretions; IgM has 10 low-affinity antigen binding domains.

In early trials of the live-attenuated oral Ty21a vaccine, seroconversion of IgG specific to LPS correlated with protection (Levine et al., 1989, Levine et al., 2001). Thus, seroconversion of immunoglobulin specific to LPS has been used to inform the development of next-generation orally-administered typhoid vaccines (Hohmann et al., 1996a, Tacket et al., 1997, Tacket et al., 2000, Tacket et al., 2004, Kirkpatrick et al., 2005).

The production of immunoglobulin specific to Vi polysaccharide, flagella and LPS has been detected in acute and convalescent serum samples (Levine et al., 1978, Calderon et al., 1986, Losonsky et al., 1987, Jesudason et al., 1998, Herath, 2003). Interestingly, however, in endemic regions, susceptibility to disease persists in the presence of raised levels of immunoglobulin to the Vi polysaccharide, flagellin and LPS (Dupont et al., 1971, Levine et al., 1978). Controlled human infection has demonstrated that there is no association between levels of serum immunoglobulin specific to these antigens prior to challenge and susceptibility to disease (Waddington et al., 2014). Immunoglobulin responses specific to flagella and LPS have been observed in volunteers susceptible to infection but not in volunteers resistant to infection (Waddington et al., 2014). In this model, no responses specific to the Vi polysaccharide have been observed in either susceptible or resistant volunteers (Waddington et al., 2014). This may be a result of the differential expression of the Vi polysaccharide *in vivo* (Tran et al., 2010a, Wilson et al., 2011).

1.10.1. Immunoglobulin function against *S*. Typhi

In regions of endemic disease, where *S*. Typhi is likely to be encountered intermittently, bactericidal immunoglobulin function has been shown to increase with age (Pulickal et al., 2009). Immunoglobulin generated through oral vaccination with attenuated *S*. Typhi has been shown to enhance direct bactericidal activity, opsonophagocytic activity and

immunoglobulin-dependent cellular cytotoxicity (Tagliabue et al., 1986, Lindow et al., 2011, Wahid et al., 2014).

IgA specific to *S.* Typhi antigens has been detected in tears, saliva, nasal wash, vaginal wash, intestinal fluids and stool following natural infection and following oral vaccination with attenuated *S*. Typhi (Forrest, 1992, Cancellieri and Fara, 1985, Herath, 2003, Pakkanen et al., 2010). IgA selectively engages M cells in order to more efficiently deliver antigen and engage adaptive responses (Rey et al., 2004, Boullier et al., 2009). Murine models have demonstrated that IgA specific to LPS prevents *S.* Typhi adherence and invasion via the intestinal mucosa (Michetti et al., 1994, Forbes et al., 2008). Indeed, in mice, secretion of IgA specific to LPS into the intestinal lumen can protect against disease (Michetti et al., 1992).

1.10.2. B cells – beyond immunoglobulin

In addition to immunoglobulin production, B cells may contribute towards immune defence through antigen presentation and cytokine production. Murine models of *Salmonella* infection have demonstrated that B cells play a prominent role in the development of protective T-cell responses (Ugrinovic et al., 2003, Nanton et al., 2012, Barr et al., 2010). B cells can present antigen via major histocompatibility complex (MHC) class II to $CD4^+$ T cells and through cross-presentation via MHC class I to $CD8^+$ T cells (Souwer et al., 2009, de Wit et al., 2010). Due to their capacity for efficacious presentation of antigen via MHC class I and MHC class II, *ex vivo* studies commonly utilise infected Epstein-Barr virus (EBV)-transformed B cells for re-stimulation of $CD4^+$ and $CD8^+$ T cells (Wahid et al., 2007, Wahid et al., 2008, Salerno-Goncalves et al., 2010, McArthur and Sztein, 2012).

Susceptibility to disease has been assessed in wild-type, B-cell-deficient, immunoglobulin-deficient and class-switched-immunoglobulin-deficient mice immunised with live-attenuated *Salmonella* (Nanton et al., 2012). It was observed that, while B-cell-deficient mice were susceptible to infection, wild-type, immunoglobulin-deficient and class-switched-

immunoglobulin-deficient mice were all, rather surprisingly, protected from infection (Nanton et al., 2012). Since immunoglobulin-deficient and class-switched-immunoglobulin-deficient mice were protected from infection, it is reasonable to hypothesise that, in mice, B-cell antigen presentation and/or cytokine production may be more important with regards the generation of protective immune responses than the direct action of immunoglobulin. It is, however, unclear whether this is the case in human disease.

1.11. T-cell-mediated immunity to *S*. Typhi

In contrast with B cells, T-cell effector function is not restricted to pathogens within the extracellular environment. T cells recognise peptides displayed by MHC molecules expressed on the surface of host-cells. MHC class I is recognised by cytotoxic $CD8^+$ T cells and is expressed on the surface of almost all cell types, it is responsible for the presentation of peptides derived from proteins sampled from within the intracellular compartment. MHC class II is recognised by $CD4^+$ T cells and is expressed on the surface of APCs, it is responsible for the presentation of peptides derived from proteins sampled from the extracellular space. Recognition of antigen by the T-cell receptor (TCR) results in activation of effector function. Memory T cells may also be generated during the course of this response; these cells are responsible for the preservation long-term T-cell-mediated immunity to the antigen recognised by the TCR.

While B-cell-mediated immunity can protect against cell invasion and disease acquisition, T-cell-mediated immune defences, which are able to target intracellular pathogens, are believed to play a key role in the clearance of established infection. The importance of T cells in the elimination of established infection has been demonstrated by murine models which have demonstrated that $CD4^+$ T-cell depletion increases the bacterial burden in the spleen (Nauciel, 1990) and results in re-emergence of latent infection (van Diepen et al., 2005).

1.11.1. T-cell cytokine production against *S*. Typhi

T-cell responses *in vitro* are influenced by the nature of the stimulant; in particular, the dependence of soluble antigenic preparations on cross-presentation to engage cytotoxic $CD8^+$ T cells. Thus, broadly speaking, $CD4^+$ T cells are more likely to respond to soluble antigenic preparations than $CD8^+$ T cells (Salerno-Goncalves et al., 2002, Salerno-Goncalves et al., 2003, Sztein, 2007).

In humans, pro-inflammatory cytokine production has been observed amongst $CD4^+$ and $CD8^+$ T cells following *ex vivo* stimulation with soluble *S*. Typhi antigens (Sztein et al., 1994, Salerno-Goncalves et al., 2002, Salerno-Goncalves et al., 2003, Salerno-Goncalves et al., 2004). Following oral vaccination with attenuated *S*. Typhi, $CD4^+$ and $CD8^+$ T cells, which produce IFN-γ in response to LPS and flagella antigens, persist in peripheral blood for up to 56 days (Sztein et al., 1994, Salerno-Goncalves et al., 2002, Salerno-Goncalves et al., 2003).

Multiphasic $CD8^+$ central memory T (T_{CM})-cell, effector memory T (T_{EM})-cell and terminal effector memory T (T_{EMRA})-cell responses, which persist for at least 2 years, have been described following oral vaccination with attenuated *S.* Typhi (Salerno-Goncalves et al., 2010). $CD8^+$ T_{CM} cells, T_{EM} cells and T_{EMRA} cells, stimulated with *S.* Typhi-infected EBV-transformed host B cells, have demonstrated the capacity to simultaneously produce as many as six cytokines/chemokines (Salerno-Goncalves et al., 2010, McArthur and Sztein, 2012). Polyfunctional T cells – those which simultaneous produce multiple cytokines/chemokines – have been shown to correlate with vaccine mediated protection against other intracellular infections (Darrah et al., 2007, Kannanganat et al., 2007). The generation of polyfunctional T cells has also been observed following environmental exposure to intracellular pathogens, including human immunodeficiency virus (HIV) and *Mycobacterium tuberculosis* (Betts et al., 2006, Caccamo et al., 2010, Qiu et al., 2012). Recently, controlled human infection has demonstrated that polyfunctional $CD8^+$ T cells are

associated with protection against disease when volunteers are challenged with approximately 10^3 CFU (Fresnay et al., 2016), but are associated with an increased susceptibility to disease when volunteers are challenged with approximately 10^4 CFU (Fresnay et al., 2017). The authors have acknowledged the relatively small sample sizes used in these studies; however, they have suggested that these observations may be attributed to the strength of the inflammatory response induced through exposure to 10^3 or 10^4 CFU (Fresnay et al., 2017). They suggest that the higher dose inoculum generates stronger inflammatory responses than the lower dose inoculum and that exposure to this inflammatory environment may favour systemic dissemination (Fresnay et al., 2017). It has been demonstrated, in murine models, that *S*. Typhi gene expression and virulence is, in part, dependent upon multiple TLR ligation (Arpaia et al., 2011). Thus, it is entirely possible that exposure to other components of the human immune system, including inflammatory factors, may influence *S*. Typhi gene expression and virulence. Further study should be undertaken to assess the validity of these hypotheses.

1.11.2. T cell cytotoxicity against *S*. Typhi

Cellular apoptosis limits the opportunity for intracellular replication and exposes *S*. Typhi to the extracellular environment and B-cell-mediated mechanisms of defence.

Oral vaccination with attenuated *S.* Typhi has been shown to generate $CD8^+$ T cells with direct cytotoxic potential (Sztein et al., 1995, Salerno-Goncalves et al., 2002, Salerno-Goncalves et al., 2003). Data indicate that the killing of *S*. Typhi-infected cells by $CD8^+$ T cells is largely mediated through granular exocytosis, through classical MHC class I and non-classical MHC class I antigen E presentation (Salerno-Goncalves et al., 2002, Salerno-Goncalves et al., 2003, Salerno-Goncalves et al., 2004). It should be noted that murine models have generated data, using in MHC class I-deficient mice, which suggests that $CD8^+$ T cells play only a modest role in the resolution of primary infection and play no role in resolution of secondary infection

(Lee et al., 2012). These data would suggest that the role of cytotoxicity is limited in the resolution of secondary infection; however, it is unclear whether this is the case in human disease.

1.11.1. Mucosal homing of T cells to *S*. Typhi

It has been suggested that the multiphasic nature of peripheral T-cell responses may be attributed to the migration of T cells to the gut and other lymphoid tissues as well as the circulation of new T cells generated in lymphoid organs. The generation of $CD4^+$ and $CD8^+$ T cells with enhanced mucosal homing potential following oral vaccination with attenuated *S*. Typhi has been observed (Lundin et al., 2002, Salerno-Goncalves et al., 2005, Wahid et al., 2008). Memory T cells which express high levels of integrin β_7 have been shown to produce approximately ten times more IFN-γ compared with memory T cells expressing low levels of integrin β_7 (Lundin et al., 2002).

Following oral vaccination with attenuated *S*. Typhi, $CD8^+$ memory T cells are generated with increased capacity for mucosal homing; they simultaneously express high levels of $\alpha_4\beta_7$, intermediate levels of CCR9 and low levels of CD103 (Salerno-Goncalves et al., 2005). It has been further demonstrated that memory $CD4^+$ and $CD8^+$ T-cell subsets possess varied capacities for mucosal homing; while most $CD4^+$ T_{CM} cells and $CD8^+$ T_{EMRA} cells express integrin $\alpha_4\beta_7$, $CD4^+$ T_{EM} cells and $CD8^+$ T_{EM} cells comprise populations which do and do not express $\alpha_4\beta_7$ (Wahid et al., 2008). The varied expression of $\alpha_4\beta_7$ is likely a reflection of the contrasting contributions these populations make towards the elimination of intestinal and systemic infection.

Interestingly, T cells, with increased mucosal homing potential, have been shown to persist in peripheral circulation for at least 90 days (Wahid et al., 2008). These cells are able to travel throughout the body via the peripheral circulatory system, where they may or may not

encounter antigen, whilst retaining their ability to rapidly migrate to the mucosa in response to infection.

1.11.1. Innate-like T cells to *S*. Typhi

Interestingly, T-cell responses to *S*. Typhi antigens are commonly observed among naïve individuals with no history of immunisation against, or infection with, the pathogen (Lundin et al., 2002, Salerno-Goncalves et al., 2010, Pennington et al., 2016). These responses may simply be the result of established adaptive immune responses generated through previous exposure to other enteric pathogens baring cross-reactive antigens; however, innate-like T cells – γδ T cells, NKT cells as well as MAIT-cells – may also contribute towards these observations (Salerno-Goncalves et al., 2014). Indeed, it has been demonstrated in humans that γδ T cells and NKT cells are able to produce larger amounts of IFN-γ than $CD4^+$ and $CD8^+$ T cells in response to stimulation with live *S*. Typhimurium (Nyirenda et al., 2010). Murine models have demonstrated that γδ T-cell depletion reduces the 50% lethal dose 300-fold (Mixter et al., 1994) and that inhibition of γδ T-cell migration to the intestinal epithelium increases disease severity (Edelblum et al., 2015).

Controlled human infection has demonstrated that, in volunteers susceptible to infection, during the course of infection, there is a sharp decline in the number of MAIT cells in peripheral blood (Salerno-Goncalves et al., 2017). Interestingly, in these volunteers, the frequency of activated MAIT cells expressing CCR9 increased significantly following diagnosis, much more so in volunteers challenged with 10^3 CFU than in volunteers challenged with 10^4 CFU (Salerno-Goncalves et al., 2017). No changes were observed in volunteers resistant to infection (Salerno-Goncalves et al., 2017). This observation may be a reflection of the strength of recruitment in volunteers challenged with the higher dose inoculum.

1.11.2. Regulatory T cells to *S*. Typhi

Regulatory T cells (T_{REG}) are able to migrate to sites of infection in order to maintain control over inflammatory immune responses through direct suppression of inflammatory effector populations and/or immunosuppressive cytokine secretion.

It has been suggested that heightened inflammation may actually favour systemic dissemination (Fresnay et al., 2017). Murine data indicate that the balance between pro-inflammatory T-cell and T_{REG} responses influences clearance of infection (Johanns et al., 2010). In humans, oral vaccination with Ty21a has been show to generate IL-10 responses (Wyant et al., 1999). Consistent with their anti-inflammatory role, depletion of T_{REG} has been shown to enhance $C8^+$ T-cell cytokine production in response to *ex vivo* stimulation with *S*. Typhi-infected EBV-transformed host B cells (McArthur et al., 2015). Controlled human infection has demonstrated that, in response to challenge, the expression of $\alpha_4\beta_7$ is reduced and other molecules associated with enhanced activation are increased among T_{REG} populations in peripheral blood in volunteers diagnosed with typhoid disease (McArthur et al., 2015). Based on these data, it is reasonable to hypothesise that T_{REG} expressing high levels of $\alpha_4\beta_7$ are depleted in peripheral blood and sequestered at the intestinal mucosa following challenge.

1.12. Innate immune memory – 'trained immunity'

Immunological studies have demonstrated that innate immune cells, upon exposure to a pathogen/vaccine, are altered in such a way as to induce qualitative and quantitative changes in response to re-exposure to identical or heterologous antigens. Thus, the dogma that only components of the adaptive immune system are able to generate immunological memory has been challenged. Indeed, evidence collected from plants (Luna and Ton, 2012, Kachroo and Robin, 2013), invertebrates (Sadd and Schmid-Hempel, 2006, Rodrigues et al., 2010) and vertebrates (Di Luzio and Williams, 1978, Tribouley et al., 1978, Krahenbuhl et al.,

1981, Bistoni et al., 1986, van der Meer et al., 1988, Bistoni et al., 1988, Vecchiarelli et al., 1989, van 't Wout et al., 1992, O'Leary et al., 2006, Barton et al., 2007, Munoz et al., 2010, Quintin et al., 2012, Marakalala et al., 2013, Ribes et al., 2014, Zhang et al., 2014, Chen et al., 2014), supports the notion that innate immune cells are also able to generate memory through a process termed trained immunity.

While adaptive immune responses are the result of receptor gene rearrangement and affinity maturation, the generation of innate immune memory is the result of epigenetic reprogramming. Typically, genes which encode inflammatory factors are packaged in a repressed configuration, inhibiting transcriptional activation (Smale et al., 2014); however, upon activation, stimulation-responsive transcription factors and promoters are recruited to these loci, resulting in increased accessibility and facilitating high-level gene expression (Ghisletti et al., 2010, Heinz et al., 2010, Barozzi et al., 2014, Smale and Natoli, 2014). Following stimulation, trained immunity is generated through a process, which is in-part dependent upon changes in cellular metabolism as well as structural histone and DNA modification (Krawczyk et al., 2010, Pantel et al., 2014). These changes facilitate enhanced transcriptional access, resulting in stronger cellular responses following re-stimulation (Ramirez-Carrozzi et al., 2006, Ramirez-Carrozzi et al., 2009). Since microRNAs (miRNAs) are known to persist for long periods with the cytoplasm and since myeloid cells have a limited proliferative capacity, miRNAs within the cytoplasm are able to persist, having long-term impact on cell function (Krol et al., 2010). Ultimately, data indicate that it is the persistence of these modifications which confers the selective advantage which has been associated with the generation of trained immunity (Foster et al., 2007).

Epidemiological evidence indicates that the Bacillus Calmette–Guérin (BCG) vaccine, the live-oral polio vaccine and measles containing vaccines all possess the capacity to confer protection against pathogens other than those targeted by those vaccines (Goodridge et al.,

2016). Immunological studies have demonstrated that monocyte phenotype is altered for at least 12 months following administration of BCG (Kleinnijenhuis et al., 2014); however, to date, no data has been presented which demonstrates that these kind of immunological observations are clinically relevant in humans (Pollard et al., 2017). Since data indicate that monocytes typically only persist in circulation for a single day (Yona et al., 2013), it is reasonable to assume that trained immunity is imprinted at the progenitor level. Due to the semi-specific nature of innate immune cell populations, it is clear how any change among these populations could broadly influence the host response to a wide array of pathogens. While trained immunity is more short-lived than traditional adaptive responses, due to the role that innate immune cell populations play in the generation of adaptive responses, the effects of trained immunity likely also influence the generation of subsequent long-lived adaptive responses.

It has been demonstrated that pre-priming of the immune system through BCG vaccination enhances functional immunoglobulin responses as well as cytokine production generated in response to influenza vaccination (Leentjens et al., 2015). Data have also been presented indicating that commensals at the mucosal surface of the gastrointestinal tract influence an individual's response to vaccination through TLR engagement (Oh et al., 2014). It has, therefore, been suggested that intestinal commensals may be considered as endogenous adjuvants. In view of these data, it is reasonable to hypothesise that a live-attenuated orally-administered vaccine, such as Ty21a, may have a similar impact to BCG via the intestinal mucosa.

1.13. Prevention of disease

Humans represent the only host and reservoir for *S*. Typhi. Inadequate sanitation and poor food hygiene facilitates faecal-oral transmission. In rare cases, sexual transmission has also been observed (Reller et al., 2003). Towards the end of the 19th century, resource-rich

nations made efforts to increase the availability of clean, or chlorinated water, and to improve sanitation infrastructure (Smith and Tennant, 1899). The implantation of these measures was associated with a marked decline in the incidence of typhoid fever (Smith, 1920). Typhoid fever continues to persist in many resource-poor nations, where access to treated water is limited and/or where living conditions are unsanitary (Figure 1.5). The risk of acquiring typhoid fever has been associated with the consumption of untreated water (Tran et al., 2005, Srikantiah et al., 2007, Ram et al., 2007). In Indonesia, individuals living in households without a toilet are at an increased risk of acquiring typhoid fever (Vollaard et al., 2004). Conversely, in Bangladesh, individuals living in households where a latrine is used are at reduced risk of acquiring typhoid fever (Ram et al., 2007).

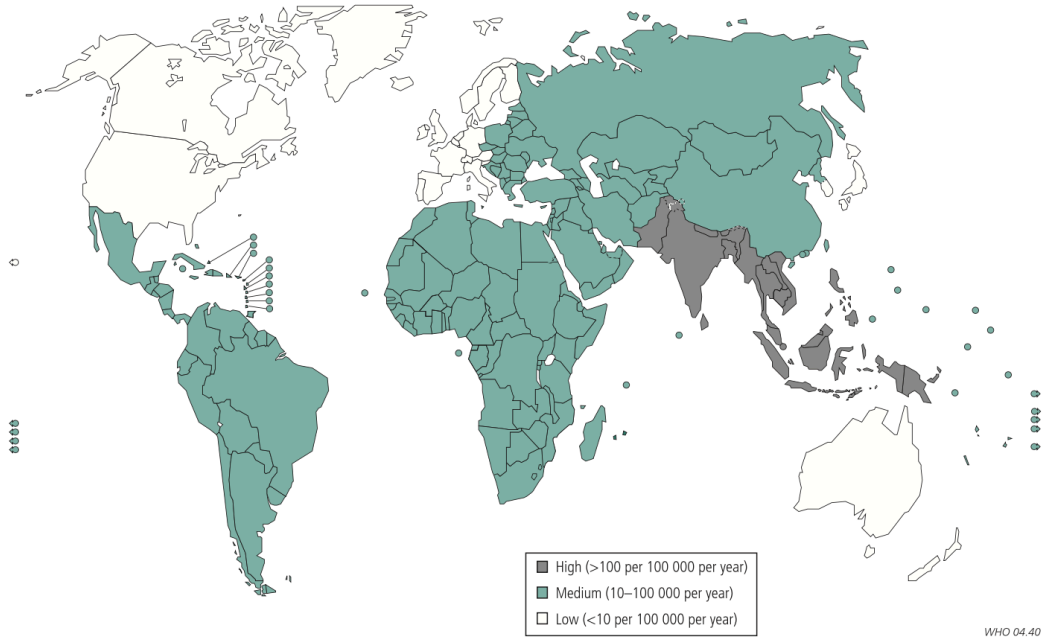

Figure 1.5. Global distribution of typhoid fever (Crump et al., 2004)

Temporal variation in the incidence of typhoid fever is thought to be the result of seasonal variation in the supply of clean water (Siddiqui et al., 2006, Crump et al., 2008). In some locations, the incidence of typhoid fever is highest during the dry season due to reduced rainfall and pressure on water sources (Velema et al., 1997, Lin et al., 2000, Crump et al.,

2008). In other locations, the incidence of typhoid fever is highest during the wet season, when the influx of large quantities of water overwhelms sanitation infrastructure leading to the contamination of clean water by sewage (Saha et al., 2001, Karkey et al., 2008).

1.14. Licensed typhoid vaccines

Since *S*. Typhi is restricted to its human host, efficacious vaccination may lead to the eradication of disease. The successful eradication of smallpox was, in part, due to human host restricted nature of the small pox virus (Metzger et al., 2015). In the face of rising antimicrobial resistance, the WHO has recommended the introduction of typhoid vaccines into vaccination programmes in endemic regions to prevent transmission and to combat outbreaks of disease (2008). To date, several typhoid vaccines have been granted licensure (Table 1.1).

1.14.1. Inactivated whole-cell

In 1896, the first inactivated whole-cell typhoid vaccine was introduced. This vaccine was administered parenterally, as a single dose of inactivated preserved preparations of wild-type *S*. Typhi in liquid suspension (Groschel and Hornick, 1981). A trial conducted in 1953 demonstrated that heat-inactivated phenol-preserved preparations were superior to alcohol-inactivated alcohol-preserved preparations (1957). The heat-inactivated phenol-preserved preparation was later shown to confer a 70% protective efficacy for at least 5 years (Ashcroft et al., 1964, Ashcroft et al., 1967).

While the whole-cell typhoid vaccine was efficacious, localised and systemic inflammatory immune responses were routinely observed; localised responses occurring in 70-80% of individuals and systemic responses occurring in 20-25% of individuals (Hejfec et al., 1966). Due, in part, to the severity of these reactions, the whole-cell typhoid vaccine was eventually withdrawn.

Table 1.1. Past and present – licensed typhoid vaccines

Vaccine type	Brand name	Manufacturer	Number of doses	Field trial efficacy	Minimum administration age
Inactivated whole cell (Injectable)	N/A	No longer in production	2	~60–80%	N/A
Live-attenuated (Oral)	Vivotif	Crucell	3-4	~60–80%	≥6 years
Vi polysaccharide (Injectable)	Typhim Vi	Sanofi Pasteur	1	~55–72%	≥2 years
	Typherix	GlaxoSmithKline	1	61%	≥2 years
	Typbar	Bharat Biotech	1	No data available	≥2 years
	Vax-TyVi	Finlay Instituto	1	No data available	≥2 years
	TyViVac	Dalat Vaccine Company	1	No data available	≥2 years
	BioTyphi	BioMed	1	No data available	≥2 years
Vi polysaccharide combination (Injectable)	Hepatyrix	GlaxoSmithKline	1	No data available	≥15 years
	ViATIM	Sanofi Pasteur	1	No data available	≥16 years
Vi polysaccharide conjugate (Injectable)	Pedatyph	BioMed	2	No data available	≥3 months
	Typbar-TCV	Bharat Biotech	1	No data available	≥6 months

Table adapted from (Sztein et al., 2014).

1.14.1. Live-attenuated oral Ty21a

In 1975, the first live-attenuated typhoid vaccine was introduced. This vaccine, designated Ty21a, was generated by non-specific chemical mutagenesis of pathogenic *S*. Typhi strain Ty2 (Germanier and Fuer, 1975). Ty21a is administered orally, in a lyophilised form via enterically coated capsules (Germanier and Fuer, 1975).

Over 20 different mutations have been identified in Ty21a (Germanier and Fuer, 1975). Amongst these, a *galE* mutation has rendered Ty21a unable to express the Vi polysaccharide. For a long time this mutation was thought to have been responsible for the attenuation of the pathogen; however, studies have since revealed that, alone, this mutation is insufficient to fully attenuate pathogenesis (Hone et al., 1988).

Ty21a was originally trialled using controlled human infection at the University of Maryland in 1977. During this trial, 5-8 oral doses were consumed at 3-4 day intervals; a single dose comprised 10^{10} viable Ty21a suspended in 45 mL milk followed by (after a 5 minute interval) 60 mL dH_2O containing 2 g $NaHCO_3$. This trial revealed that, when cultured in the presence of an exogenous source of galactose, Ty21a conferred a 87% protective efficacy (Gilman et al., 1977).

Two subsequent field trials demonstrated the variable efficacy of this vaccine; a 96% protective efficacy was demonstrated after 3 years in Egypt (1978-1981) (Wahdan et al., 1982) and a 67% protective efficacy was demonstrated after 3 years in Chile (1983-1986) (Levine et al., 1987). More recently, a systematic review calculated a cumulative efficacy of 58% up to 2 years following vaccination (Anwar et al., 2014). By virtue of there being less individuals susceptible to infection, rates of transmission are reduced and additional benefits associated with the generation of herd immunity have been observed (Levine et al., 1989).

The safety of Ty21a is well established (Gilman et al., 1977); however, limitations do exist. Vaccination is dependent upon the administration of three spaced doses and the vaccine

possesses only modest immunogenicity (Anwar et al., 2014). In addition, children in the developing world tend to be hyporesponsive to orally-administered vaccines (Levine et al., 1987, Olanratmanee et al., 1992); however, it has been demonstrated that when administered in liquid suspension, Ty21a is immunogenic in children aged between 2 and 6 years (Olanratmanee et al., 1992, Bhuiyan et al., 2014).

1.14.2. Purified Vi polysaccharide

In 1994, following successful trials, the first subunit typhoid vaccine was introduced (Keitel et al., 1994). This vaccine is administered parenterally as a single dose of purified Vi polysaccharide in liquid suspension.

Early trials demonstrated that purified Vi polysaccharide alone was unable to confer protection against disease (Gaines et al., 1961). As a result, the pursuit of Vi polysaccharide based vaccines was abandoned. Interest was reignited when evidence was presented which demonstrated that expression of the Vi polysaccharide enhanced the protective efficacy of heat-killed vaccine preparations (Wong et al., 1974). It was later determined that conformational changes brought about during isolation of the polysaccharide were responsible for previous vaccine failure (Landy et al., 1961). A refined method of polysaccharide isolation, which was developed for the generation of vaccines targeting meningococcus (Gotschlich et al., 1969), was later used to isolate and develop an effective Vi polysaccharide based vaccine targeting typhoid fever.

Two large-scale field trials have demonstrated the efficacy of this vaccine; a 75% protective efficacy was demonstrated over 17 months in Nepal (Acharya et al., 1987) and a 77% protective efficacy over 21 months in South Africa (Klugman et al., 1987). A recent systematic review calculated a cumulative efficacy of 55% 3 years following vaccination (Anwar et al., 2014). Additional benefits associated with the generation of herd immunity have been observed (Sur et al., 2009).

Decline in the levels of circulating immunoglobulin over the 3 years following administration of the Vi polysaccharide vaccine has been observed and there are no data which suggest that efficacy persists beyond 3 years (Froeschle and Decker, 2010). As is the case for all purified polysaccharide vaccines, immune responses are T-cell independent; thus, no immune memory is generated and responses may not be boosted through repeated vaccination (Lesinski and Westerink, 2001). Although trials have not been conducted in young children, vaccination with purified Vi polysaccharide is unlikely to be efficacious due to the immaturity of the splenic marginal zone which is required for the generation of T-cell independent responses (Pulickal and Pollard, 2007).

1.15. Next-generation typhoid vaccines

Interventions aimed at improving the availability of clean drinking water and programmes which provide education in sanitation and food hygiene could dramatically reduce the burden of typhoid fever in endemic regions (2008). Unfortunately, the implementation of these measures is occurring at a discouragingly slow pace. Thus, due to the successful implementation of mass vaccination in areas of endemic disease (Bodhidatta et al., 1987), efforts have been directed towards the development of new, more effective vaccines, which may be more easily delivered to areas where they are most needed (Table 1.2).

Table 1.2. Typhoid vaccines in development

Vaccine type	Name	Manufacturer	Number of doses	Field trial efficacy	Minimum administration age
Live-attenuated (Oral)	CVD 908	University of Maryland	1	No data available	No data available
	CVD 908-*htrA*	University of Maryland	1	No data available	No data available
	CVD 909	University of Maryland	1	No data available	No data available
	Ty800	University of Maryland	1	No data available	No data available
	M01ZH09	Massachusetts General Hospital	1	No data available	No data available
Vi-conjugate (Injectable)	Vi-TT	BioMed	1	100%	≥6 months
	Vi-rEPA	NIH	2	89%	≥2 years
	Vi-CRM_{197}	Novartis Vaccines Institute	1	No data available	≥2 years

Table adapted from (Sztein et al., 2014).

1.15.1. Next-generation live-attenuated orally-administered vaccines

Orally-administered vaccines are able to generate responses tailored to defence at the intestinal mucosa. The orally-administered Ty21a vaccine is more well-tolerated clinically and easier to administer than parenterally-administered alternatives (Anwar et al., 2014). Ty21a is, however, limited by its modest immunogenicity and dependence upon multiple spaced doses to confer protection (Anwar et al., 2014). A number of live-attenuated orally-administered typhoid vaccines are currently in development, which are intended to be as well-tolerated as Ty21a, yet more immunogenic and effective as a single dose.

CVD 909

CVD 909 is a refinement of CVD 908-*htrA* and its predecessor CVD 908. CVD 908 was engineered from pathogenic *S*. Typhi strain Ty2 through deletion of *aroC* and *aroD* (Tacket et al., 1992). The Δ*aroC*/Δ*aroD* double mutation prevents synthesis of chorismic acid, rendering the bacteria auxotrophic for *p*-aminobenzoic acid and 2,3-dihydroxybenzoate, compounds not available to the bacteria within mammalian tissue (Tacket et al., 1992). CVD 908 has been shown to be well-tolerated and immunogenic; however, its administration was associated with a silent, self-resolving vaccinaemia (Tacket et al., 1992).

In an effort to prevent vaccinaemia, CVD 908 was further attenuated; CVD 908-*htrA* was engineered through deletion of *htrA*. The Δ*htrA* mutation prevents expression of a heat shock protein involved in intracellular survival and replication (Johnson et al., 1991). CVD 908-*htrA* has been shown to be as immunogenic CVD 908, but, crucially, was not associated with vaccinaemia (Tacket et al., 2000, Salerno-Goncalves et al., 2003).

Since the parenterally-administered Vi polysaccharide vaccine does confer a moderate level of protection against disease (Anwar et al., 2014), efforts were made to enhance immunity generated to this antigen by CVD 908-*htrA*; CVD 909 was engineered to constitutively express the Vi polysaccharide by switching the *tviA* promoter, which regulates Vi polysaccharide

expression, with the constitutive *tac* promoter. CVD 909 has been shown to generate an enhanced response to the Vi polysaccharide (Tacket et al., 2004). IgG responses specific to the Vi polysaccharide have been observed in 80% of volunteers receiving 1 or 2 doses of 10^6 – 10^9 CFU CVD 909. It is significant that these responses may be boosted through subsequent administration of the parenterally-administered purified Vi polysaccharide vaccine, since this demonstrates that CVD 909 generates memory B cells specific to the Vi polysaccharide (Wahid et al., 2011).

Ty800

Ty800 was engineered from pathogenic *S*. Typhi strain Ty2 through deletion of *phoP* and *phoQ* (Hohmann et al., 1996b). The Δ*phoP*/Δ*phoQ* double mutation inhibits expression of multiple virulence factors, including those which facilitate intracellular survival (Hohmann et al., 1996b). Ty800 has been shown to be well-tolerated and immunogenic (Hohmann et al., 1996a). IgG responses specific to *S.* Typhi antigens were observed in 81% of volunteers vaccinated with Ty800 and in 62.5% of volunteers vaccinated with Ty21a (Hohmann et al., 1996a). The development of this promising candidate has not been pursued.

M01ZH09

M01ZH09 was engineered from pathogenic *S*. Typhi strain Ty2 through deletion of *aroC and ssaV* (Kirkpatrick et al., 2005). The Δ*aroC* mutation disrupts synthesis of chorismic acid, rendering the bacteria auxotrophic for 2,3-dihydroxybenzoate, a compound which is not available to the bacteria within mammalian tissue. The Δ*ssaV* mutation disrupts expression of SPI-2 which impacts intracellular survival (Coombes et al., 2005, Kirkpatrick et al., 2006, Lyon et al., 2010). M01ZH09 has been shown to be well-tolerated and immunogenic in both adults and children (Kirkpatrick et al., 2006, Tran et al., 2010b). In one trial, children aged between 5 and 14 years were given 5×10^9 CFU M01ZH09. Seroconversion, defined as a 1.7-fold increase in levels of IgG and a 1.5-fold increase in levels of IgA specific to LPS, was

observed in 97% of vaccinated volunteers (Tran et al., 2010b). Controlled human infection has since demonstrated that, despite its immunogenicity, administered as a single dose, M01ZH09 has a protective efficacy of just 13% (Darton et al., 2016). In the same trial, Ty21a was estimated to have a protective efficacy of just 35% (Darton et al., 2016). This is consistent with the calculated cumulative efficacy of Ty21a of 35% up to 1 year following vaccination (Anwar et al., 2014).

1.15.2. Next-generation protein conjugate parenterally-administered vaccines

A number of parenterally-administered Vi polysaccharide protein conjugate vaccines are currently in development. The conjugation of polysaccharide to protein facilitates T-cell engagement, enabling immunoglobulin affinity maturation, immunoglobulin isotype switching and the development immunological memory in both adults and children (Levine et al., 2001, Pulickal and Pollard, 2007). The development of this technology has revolutionised vaccine development.

Conjugation of the Vi polysaccharide to a nontoxic, recombinant protein – *Pseudomonas aeruginosa* exotoxin – was first described in 1987 (Szu et al., 1987). Administration of multiple doses of Vi polysaccharide protein conjugate vaccines enhances immunity and does not result in immune hypo-responsiveness, which is associated with administration of multiple doses of unconjugated Vi polysaccharide vaccines (An et al., 2012). Based upon these data, a number of pharmaceutical companies have developed Vi polysaccharide protein conjugate vaccines using a number of different carrier proteins, which are at various stages of development (Table 2) (van Damme et al., 2011, Thiem et al., 2011).

Vi-rEPA (Pseudomonas aeruginosa) conjugate

Vi-rEPA was developed by linking the Vi polysaccharide to *P. aeruginosa* exoprotein A. Dose-immunogenicity studies have demonstrated that administration of 25 μg Vi-rEPA produces

stronger immunoglobulin responses than administration of 12.5 µg or 5 µg Vi-rEPA (Canh et al., 2004). In one trial, 2 doses of 22.5 µg Vi-rEPA, administered 6 weeks apart, was shown to be well-tolerated with a 89% protective efficacy in children aged between 2 and 5 years, over 46 months (Lin et al., 2001, Mai et al., 2003). Levels of IgG specific to the Vi polysaccharide increased by at least 10-fold in all vaccinated volunteers 4 weeks following administration of the second dose of the vaccine (Lin et al., 2001). It was also observed that, in vaccinated volunteers who developed disease, severity was lessened (Lin et al., 2001, Mai et al., 2003).

Vi-rEPA has also been shown to be well-tolerated and immunogenic in infants (Thiem et al., 2011). Specifically, it was demonstrated that 4 doses of 25 µg Vi-rEPA, administered at 2, 4, 6 and 12 months of age, increased levels of IgG specific to the Vi polysaccharide above the estimated protective level in 95% of infants 1 month following administration of the fourth dose (Thiem et al., 2011). In this trial, Vi-rEPA was administered alongside Expanded Programme on Immunisation (EPI) vaccines and no detrimental impact on EPI vaccines was reported (Thiem et al., 2011).

Vi-CRM$_{197}$ (diphtheria toxoid) conjugate

Vi-CRM$_{197}$ was developed by linking the Vi polysaccharide to diphtheria toxoid (CRM$_{197}$). This vaccine has been shown to be well-tolerated and immunogenic in adults (18 to 45 years), children (24 to 59 months), older infants (9 to 12 months) and infants (6 to 8 weeks) (Bhutta et al., 2014). Levels of IgG specific to the Vi polysaccharide significantly increased in adults, children and older infants 1 months following administration of 1 dose of 5 µg Vi-CRM$_{197}$ (Bhutta et al., 2014). Significant responses were also observed in younger infants but the levels of IgG were consistently lower than those observed in older age groups (Bhutta et al., 2014). In children and older infants, a second dose of 5 µg Vi-CRM$_{197}$ was administered 2 months following administration of the first; however, levels of immunoglobulin were not increased further (Bhutta et al., 2014). It should also be noted that, in both adults and

children, 6 months following administration of the final dose, levels of immunoglobulin in volunteers vaccinated with Vi-CRM_{197} were similar to those in volunteers vaccinated with unconjugated purified Vi polysaccharide (Bhutta et al., 2014). In infants and older infants, Vi-CRM_{197} was administered alongside EPI vaccines and no significant detrimental impact on EPI vaccines was reported (Bhutta et al., 2014).

Vi-TT (tetanus toxoid) conjugate

Vi-TT was developed by linking the Vi polysaccharide to tetanus toxoid. In one trial, 2 doses of 5 μg Vi-TT, administered 6 weeks apart, was shown to be well-tolerated with a 100% protective efficacy in children aged between 6 months and 12 years, over 12 months (Mitra et al., 2016). Seroconversion, defined as a 4-fold increase in levels of IgG specific to the Vi polysaccharide, was observed in 100% of vaccinated volunteers at 6 weeks and 83% of vaccinated volunteers at 12 months (Mitra et al., 2016).

More recently, controlled human infection has been used to assess the efficacy of Vi-TT. When a comprehensive all-encompassing definition of typhoid was applied, Vi-TT was estimated to have a 54.6% protective efficacy (Jin et al., 2017); however, when a broader definition was used, more in line field trial definitions, Vi-TT was estimated to have a 87.1% protective efficacy (Jin et al., 2017). This estimate is similar to the field trial efficacy of Vi-rEPA (Lin et al., 2001, Mai et al., 2003), but less than that reported in field trials of Vi-TT (Mitra et al., 2016). Interestingly, as was the case for Vi-rEPA vaccinated volunteers (Lin et al., 2001, Mai et al., 2003), it was observed that disease severity was lessened in Vi-TT vaccinated volunteers (Jin et al., 2017).

The implications of Vi polysaccharide protein conjugate vaccine efficacy are profound. The adoption of these vaccines, which possess efficacy in those most at risk of disease, into EPI could well lead to the eradication of *S*. Typhi as a cause of human disease.

1.15.3. Vaccines with broad serovar coverage

Vi polysaccharide-based vaccines may only target serovars which express this antigen. Of the more than 2,600 *S. enterica* serovars, only *S*. Typhi, *S*. Paratyphi C and *S*. Dublin express the Vi-polysaccharide (Hashimoto and Khan, 1997). In addition, Vi polysaccharide negative strains of *S*. Typhi have been isolated from individuals living in India and Nepal diagnosed with typhoid fever (Mehta and Arya, 2002, Wain et al., 2005). Thus, since pathogenicity is not dependent upon expression of the Vi polysaccharide, vaccines targeting this antigen may simply apply pressure for the selection of Vi-negative strains of *S*. Typhi.

The identification of protein antigens which are conserved across serovars could lead to the development of a universal *Salmonella* vaccine, targeting both typhoidal and non-typhoidal serovars. It has already been demonstrated that vaccination with live-attenuated *S*. Typhi generates cross-reactive B-cell and T cell responses with efficacy against a range of typhoidal (Wahid et al., 2014, Pakkanen et al., 2015, Wahid et al., 2016) and non-typhoidal serovars (Kantele et al., 2012, Pakkanen et al., 2015). In humans, vaccination with outer membrane proteins (OMPs) from *S*. Typhi has been shown to be well-tolerated and capable of generating both B-cell and T cell responses (Salazar-Gonzalez et al., 2004). Murine models have demonstrated that responses generated though vaccination with OMPs from *S*. Typhi can confer protection against a range of non-typhoidal serovars (Tabaraie et al., 1994, Isibasi et al., 1988). Further work would be required to identify viable immunogenic antigens which may be used to provide broad serovar coverage.

1.16. Aims and objectives of this thesis

This thesis aims to assess the human immune response to live-attenuated *S*. Typhi (Ty21a) through the following objectives:

- To characterise the short-term impact of Ty21a on adaptive human immunity in peripheral blood and at the duodenal and colonic mucosa

- To characterise the long-term impact of Ty21a on adaptive human immunity in peripheral blood and at the duodenal mucosa
- To characterise the non-specific effects of Ty21a on innate cell phenotype and its impact on immune responses to an array of pathogens

CHAPTER 2: MATERIALS AND METHODS

This chapter sets out the methodologies used to generate the data which are presented in subsequent chapters.

2.1. Ethical approval

All studies were compliant with Good Clinical Practice (GCP) and with the principles set out in the Declaration of Helsinki (World Medical, 2013). All studies were approved by local Research Ethics Committees (RECs) which are part of the United Kingdom National Research Ethics Service (NRES) (Table 2.1).

Table 2.1. Ethical approval and REC information

Project title	REC	Reference	Results
Mucosal Responses to Oral Vaccination	North West – Liverpool Central	10/H1005/20	Chapter 3
Understanding Immunity to Typhoid	North West – Liverpool Central	13/NW/0282	Chapter 4
Non-specific Effects of Typhoid Vaccination	North West – Liverpool East	14/NW/1455	Chapter 5

2.2. Recruitment and Consent

All volunteers provided written informed consent. Volunteers were recruited by electronic and poster advertisement at the University of Liverpool, the Liverpool School of Tropical Medicine and the Royal Liverpool University Hospital. All volunteers were screened according to strict inclusion and exclusion criteria (Table 2.2). These criteria were in place to ensure that volunteers were able to provide full informed consent and to minimise variability within the cohort.

Table 2.2. Study inclusion and exclusion criteria

Study reference	Criteria	
Chapter 3	Inclusion	Have capacity to give informed consent Are aged between 18 and 60 years Are in good health Are fluent in English
	Exclusion	Are currently involved in another study Are taking medication that affects the immune system Have smoked more than 20 cigarettes a day for 10 years Are currently pregnant Are not fluent in English *Additional vaccine group criteria:* *Previous adverse reaction to vaccination*
Chapter 4	Inclusion	Have capacity to give informed consent Are aged between 18 and 60 years Are in good health Are fluent in English
	Exclusion	Are currently involved in another study Are taking medication that affects the immune system Are currently pregnant Are not fluent in English *Additional control group criteria:* *Have visited or lived in a typhoid endemic region* *Previously vaccinated against typhoid*
Chapter 5	Inclusion	Have capacity to give informed consent Are aged between 18 and 60 years Are in good health Are fluent in English
	Exclusion	Are currently involved in another study Are taking medication that affects the immune system Are currently pregnant Are not fluent in English Have a chronic illness Have visited or lived in a typhoid endemic region Previously vaccinated against typhoid

2.3. Vaccination

Volunteers were vaccinated with Ty21a (Vivotif; Crucell). Volunteers were instructed to consume a single enteric-coated vaccine capsule (Table 2.3) on days 0, 2 and 4, approximately one hour before a meal, with a cold or lukewarm drink, according to the manufacturer's instructions.

Table 2.3. Typical composition of a single Vivotif vaccine capsule

Component	Quantity
Viable *S.* Typhi (Ty21a)	$2.0 – 10.0 \times 10^9$ CFU
Non-viable *S.* Typhi (Ty21a)	$5.0 – 50.0 \times 10^9$ CFU
Sucrose	3.3 – 34.2 mg
Ascorbic acid	0.2 – 2.4 mg
Amino acid mixture	0.3 – 3.0 mg
Lactose	Up to 180 – 200 mg
Magnesium stearate	3.6 – 4.0 mg

2.4. Clinical procedures

2.4.1. Clinical consumables

Table 2.4. Clinical consumables for sample collection

Name	Manufacturer	Product code
BD Vacutainer Safety-Lok	BD Biosciences	368654
S-Monovette 7.5 mL Z	Sarstedt	01.1601.001
BD Vacutainer 10 mL	BD Biosciences	367874
Vacuette 9 mL	Greiner Bio-One	455084
Single-Use Radial Jaw 4 Large Capacity Forceps	Boston Scientific	M00513320
133 mL Fleet Phosphate Liquid Enema	Fleet Laboratories	2027266

2.4.2. Venesection and peripheral sampling

Peripheral blood samples were collected from the antecubital fossa using a BD Vacutainer Safety-Lok blood collection set. The sampling site was prepared using isopropyl alcohol wipes and universal precautions used. Blood samples were collected in clotting activator tubes for serum isolation and heparinised tubes for isolation of peripheral blood mononuclear cells (PBMCs) (Table 2.5).

Table 2.5. Peripheral blood collection tubes

Study reference	Name	Description
Chapter 3	S-Monovette 7.5 mL Z	Clotting activator for serum isolation
	BD Vacutainer 10 mL	Sodium heparin for PBMC isolation
Chapter 4	S-Monovette 7.5 mL Z	Clotting activator for serum isolation
	Vacuette 9 mL	Lithium heparin for PBMC isolation
Chapter 5	S-Monovette 7.5 mL Z	Clotting activator for serum isolation
	Vacuette 9 mL	Lithium heparin for PBMC isolation

2.4.3. Endoscopy and mucosal sampling

Volunteers fasted from midnight the evening before their procedure. Volunteers arrived at the Department of Gastroenterology at the Royal Liverpool University Hospital for endoscopy at approximately 0800 hours. Consent for endoscopic procedures was reconfirmed by the endoscopist, according to standard National Health Service (NHS) procedures. Sedation was offered to all volunteers; those who requested sedation were given up to 5 mg midazolam intravenously. O_2 was administered at a rate 2 L/minute via a nasal cannula and O_2 saturation was monitored throughout the procedure using a pulse oximeter.

Gastroscopy

12-15 single-bite biopsies were acquired from the duodenum at D2-D3 using Single-Use Radial Jaw 4 large capacity forceps. Biopsies were placed directly into 50 mL Falcon tubes containing 40 mL R15 medium (Table 2.11) and stored on ice pending laboratory sample processing. Following the procedure, volunteers were observed for up to two hours, given a drink and a snack and then discharged.

Sigmoidoscopy

A 133 mL Fleet Phosphate Liquid Enema was administered 20 minutes prior to the procedure. 12-15 single-bite biopsies were acquired from the sigmoid colon at an insertion distance of 20-25 cm using Single-Use Radial Jaw 4 large capacity forceps. Biopsies were placed directly

into 50 mL Falcon tubes containing 40 mL R15 medium (Table 2.11) and stored on ice pending laboratory sample processing. Following the procedure, volunteers were observed for two hours, given a drink and a snack and then discharged.

2.5. Laboratory procedures

2.5.1. Laboratory consumables

Table 2.6. General laboratory consumables

Name	Manufacturer	Product code
96-well plates (sterile non-treated)	Corning	3367
50 mL Falcon tubes	Corning	352070
15 mL Falcon tubes	Corning	352095
70 µm cell strainers	Corning	352350
LeucoSep centrifuge tubes	Greiner Bio-One	227290
Sterile treated 24-well plates	Greiner Bio-One	662160
Cryovials	Fisher Scientific	50001020
Mr. Frosty freezing container	Fisher Scientific	51000001
Pasteur pipettes	StarLab	E14140311
Reagent reservoirs	StarLab	E23101010
16-gauge blunt-ended needles	Stemcell	28110

Table 2.7. Consumables for ELISA

Name	Manufacturer	Product code
Anti-human-IgA	AbD Serotec	205008
Streptavidin:alkaline phosphotase	AbD Serotec	STAR6B
96 well flat-bottom microtitre plates	NUNC	442404
Lyophilised bovine serum albumin (BSA)	Sigma-Aldrich	A2153
Anti-human-IgG	Sigma-Aldrich	A3188
Carbonate-bicarbonate capsules	Sigma-Aldrich	C3041
Lyophilised *S.* Typhi LPS	Sigma-Aldrich	L2387
Tween-20	Sigma-Aldrich	P1754
Phosphate buffered saline (PBS) tablets	Sigma-Aldrich	P4417
***p*-nitrophenyl phosphate**	Sigma-Aldrich	

Table 2.8. Consumables for tissue culture

Name	Manufacturer	Product code
Lymphoprep	Axis-Shield	1114544
CTL-Cryo ABC media	Cellular Technology	CTLABC300
96-well plates (sterile treated)	Corning	3894
Ficoll-Paque PLUS	GE Healthcare	11778538
Hanks' balanced salt solution (Ca^-/Mg^-) ($HBSS^{-/-}$)	GE Healthcare	11546291
Foetal bovine serum (FBS)	Invitrogen	10270106
Dulbecco's PBS Ca^-/Mg^- ($PBS^{-/-}$)	Invitrogen	14190169
Antibiotic/antimitotic	Invitrogen	15240062
0.5 M EDTA	Invitrogen	15575020
RPMI-1640 (+ L-glutamine + HEPES)	Invitrogen	22400089
Histopaque-1077	Sigma-Aldrich	10771
Collagenase	Sigma-Aldrich	C1764
DNase I	Sigma-Aldrich	DN25
Trypan Blue 0.4%	Sigma-Aldrich	T5154

Table 2.9. Consumables for cytokine detection by flow cytometry

Name	Manufacturer	Product code
Cytofix/Cytoperm	BD Biosciences	554714
Perm/Wash	BD Biosciences	554723
BD GolgiStop	BD Biosciences	554724
BD GolgiPlug	BD Biosciences	555029

2.5.2. Reagent preparation

Table 2.10. Reagents for ELISA

Reagent	Preparation
PBS	Five PBS tablets were dissolved in 1 L of sterile, pyrogen free water (dH_2O). Once prepared, PBS was stored at room temperature for up to 1 month.
PBS-tween	500 µL of Tween-20 was added to 1 L of PBS. Once prepared, PBS-tween was stored at room temperature for up to 1 month.
Carbonate-bicarbonate buffer	The contents of five carbonate-bicarbonate capsules were dissolved in 500 mL of dH_2O. Once prepared, carbonate-bicarbonate buffer was stored at 4˚C for up to 1 month.
1.0% BSA	5 g of lyophilised BSA was dissolved in 500 mL of PBS. Once prepared, 1.0% BSA was stored at 4˚C for up to 1 month.
0.1% BSA	50 mL of 1.0% BSA was added to 450 mL of PBS. Once prepared, 0.1% BSA was stored at 4˚C for up to 1 month.
***S*. Typhi LPS**	10 mg lyophilised *S*. Typhi LPS was suspended in dH_20 at a concentration of 5 mg/mL. Once prepared, the LPS antigen was stored at 4˚C.
***p*-nitrophenyl phosphate**	One single *p*-nitrophenyl phosphate tablet was dissolved in 40 mL dH_2O.

Table 2.11. Reagents for tissue culture

Reagent	Preparation
Complete medium	50 mL FBS was added to 450 mL RPMI-1640 (+ L-glutamine + HEPES). Once prepared, complete medium was stored at 4˚C for up to one week.
DNase I medium	50 mL FBS and 5 mL DNase I (2 mg/mL) was added to 445 mL RPMI-1640 (+ L-glutamine + HEPES). DNase I medium was used on the day of preparation.
R15 medium	75 mL FBS and 5 mL antibiotic/antimitotic (10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin and 25 µg/mL of Gibco Amphotericin B) was added to 420 mL RPMI-1640 (+ L-glutamine + HEPES). Once prepared, R15 medium was stored at 4˚C for up to one week.
R20 medium	100 mL FBS and 5 mL antibiotic/antimitotic was added to 395 mL RPMI-1640 (+ L-glutamine + HEPES). Once prepared, R20 medium was stored at 4˚C for up to one week.
CII-S medium	50 mg collagenase was added to 100 mL MR15 medium. CII-S medium was used on the day of preparation.
Wash buffer	10 mL FBS and 2 mL 0.5 M EDTA was added to 488 mL Dulbecco's $PBS^{-/-}$. Once prepared, wash buffer was stored at 4˚C for up to one week.

2.5.3. Bacterial dilution plating

The bacterial source was serially diluted 1:10 across a range from 10^1 to 10^6 in Dulbecco's $PBS^{-/-}$ in sterile untreated 96-well plates. LB-agar plates were divided into four sections, which were labelled 10^3, 10^4, 10^5 and 10^6. Three 10 µL spots were added to each of the corresponding sections and the plate allowed to dry. Plates were inverted and incubated overnight at 37°C in 5% CO_2. The next day the largest number of countable colonies in a single section were enumerated and the following equation used to calculate CFU/µL:

$$\left[\left(\frac{Number\ of\ colonies\ counted}{Suspension\ volume\ plated}\right) \times Dilution\ factor\right] = CFU/\mu L$$

2.5.4. Serum isolation

Peripheral blood samples were collected in clot activator collection tubes (Table 2.5). Samples were left at room temperature for no less than 30 minutes. Clotted samples were centrifuged at 1,300 g for 5 minutes and the serum supernatant aliquoted into Eppendorf tubes in 100 µL volumes and stored at -80˚C.

2.5.5. PBMC isolation

Chapter 3 and Chapter 4

Peripheral blood samples were collected in heparinised collection tubes (Table 2.5). Samples were diluted with an equal volume of Dulbecco's PBS and layered on either Lymphoprep (Chapter 3) or Histopaque-1077 (Chapter 4) in 50 mL Falcon tubes. Samples were then centrifuged at 800 g for 18 minutes at room temperature without brake. Cells were isolated using a sterile Pasteur pipette, washed twice (centrifuged at 250 g for 10 minutes) with Dulbecco's $PBS^{-/-}$ and resuspended in 1mL complete medium. A sample of the cell suspension was diluted 1:1 by adding 10 µL of the cell suspension to 10 µL 0.4% Trypan Blue. The number of viable cells was then enumerated using an Improved Neubauer Haemocytometer (CamLab; 1127884). The following equation was used to calculate cells/mL:

$$(Number\ of\ cells\ counted \times Dilution\ factor) \times 10^4\ = cells/mL$$

The concentration of the cell suspension was adjusted with complete medium to 1 × 10^7 cells/mL. 100 µl of the cell suspension was then added to each well of a 96-well plate, as required.

Chapter 5

Peripheral blood samples were collected in heparinised collection tubes (Table 2.5). Samples were layered on Ficoll-Paque PLUS using LeucoSep centrifuge tubes and centrifuged at 800 g for 18 minutes at room temperature without brake. Cells were isolated using a sterile Pasteur pipette, washed twice (centrifuged at 250 g for 10 minutes) with $HBSS^{-/-}$ and resuspended in 10mL $HBSS^{-/-}$. A sample of the cell suspension was diluted 1:1 by adding 10 µL of the cell suspension to 10 µL 0.4% Trypan Blue. The number of viable cells was then enumerated, as previously described.

Cells were stored using CTL-Cryo ABC media. Cells were washed once (centrifuged at 250 g for 10 minutes) with $HBSS^{-/-}$ and the cell pellet resuspended in CryoC at a concentration of 2 × 10^7 cells/mL. Samples were then placed on ice and an equal volume of CryoA:CryoB (4:1) was slowly added to achieve a final concentration of 1 × 10^7 cells/mL. The cell suspension was aliquoted into cryovials in 1 mL volumes. Cryovials were then placed in a cooled Mr. Frosty freezing container and transferred to -80˚C. After 24 hours cryovials were transferred on dry ice to -200˚C storage.

2.5.6. PBMC thawing and overnight 'rest'

Chapter 5

Cells were removed from -200˚C storage and incubated at 37°C for 10 minutes. Cryovials were inverted twice and the contents of the cryovial transferred into 15 mL Falcon tubes. Cryovials were washed once with 1 mL prewarmed DNase I medium in order to collect any remaining cells. An additional 8 mL prewarmed DNase I medium was added to the 15 mL

Falcon tubes at a rate of 1 mL every five seconds. Samples were washed twice (centrifuged at 330 g for 10 minutes) with prewarmed DNase I medium and resuspended in 600 µL prewarmed complete medium. A sample of the cell suspension was diluted 1:1 by adding 10 µL of the cell suspension to 10 µL 0.4% Trypan Blue. The number of viable cells was then enumerated, as previously described.

The concentration of the cell suspension was adjusted with complete medium to 1×10^7 cells/mL. 100 µL of the cell suspension was then added to each well of a 96-well plate, as required. Cells were then 'rested' overnight at 37°C in 5% CO_2. The following day, plates were centrifuged at 330 g for 10 minutes, the supernatant removed and the cells in each well resuspended in 100 µL prewarmed complete medium.

2.5.7. Mucosal mononuclear cell (MMC) isolation and overnight 'rest'

Samples were centrifuged at 400 g for 10 minutes at room temperature without brake. The supernatant was removed and the biopsies resuspended in 20 mL prewarmed CII-S medium and placed at 37°C for 30 minutes at a 45° angle with shaking (220 rpm). Biopsies were mechanically disrupted by passing the tissue suspension through a 16-gauge blunt-ended needle five times. The suspension was then passed through a 70 µm cell strainer and the cell suspension stored on ice. Tissue fragments captured in the cell strainer were transferred back into the original tube by rinsing the cell strainer with prewarmed CII-S medium. The process of incubation, disruption and filtration was then repeated twice more. Cell suspensions were pooled and centrifuged at 400 g for 10 minutes without brake. Cells were resuspended in 1 mL R20 medium. A sample of the cell suspension was diluted 1:1 by adding 10 µL of the cell suspension to 10 µL 0.4% Trypan Blue. The number of viable cells was then enumerated, as previously described. The concentration of the cell suspension was adjusted

with R20 medium to 1 × 10^6 cells/mL. The entire cell suspension was then distributed, in 2 mL volumes, in 24-well plates. Samples were then rested overnight at 37°C in 5% CO_2.

The following day, cells were harvested from wells and the wells washed with 1 mL R20 medium. Cell suspensions were then centrifuged at 400 g for 10 minutes and resuspended in 1 mL complete medium. A sample of the cell suspension was diluted 1:1 by adding 10 µL of the cell suspension to 10 µL 0.4% Trypan Blue. The number of viable cells was then enumerated, as previously described. The concentration of the cell suspension was adjusted with complete medium to no less than 0.5 × 10^7 cells/mL. 100 µl of the cell suspension was then added to each well of a 96-well plate, as required.

2.5.8. Phenotypic analysis of 'rested' monocytes

Cells were washed (centrifuged at 330 g for 10 minutes) with 200 µL wash buffer. Cells were re-suspended in 50 µL wash buffer containing viability dye.

Table 2.12. Viability dye for the analysis of monocyte phenotype

Study reference	Name	Description	Manufacturer	Per test
Chapter 5	LIVE/DEAD	Pacific Orange	LifeTechnologies	0.02 µL

Cells were incubated for 20 minutes at room temperature in the absence of light. 150 µL wash buffer was added to each well and the plates centrifuged at 330 g for 10 minutes. Cells were resuspened in 50 µL wash buffer containing the following antibodies:

Table 2.13. Surface marker antibody panel for analysis of monocyte phenotype

Study reference	Target	Clone	Fluorophore	Manufacturer	Per test
Chapter 5	CD14	MϕP9	PerCP/Cy5.5	BD Biosciences	3.5 µL
	CD16	FcγRIII	APC/Cy7	BioLegend	3.5 µL
	CD64	HI10a	BV605	BioLegend	3.5 µL
	CD18	TS1/18	PE/Cy7	BioLegend	3.5 µL
	CD11b	ICRF44	AF700	BD Biosciences	3.5 µL
	CD11c	Bu15	Pacific Blue	BioLegend	3.5 µL
	CD123	6H6	BV711	BioLegend	3.5 µL
	CD206	19.2	PE/CF594	BD Biosciences	3.5 µL
	CD284	HTA125	APC	BioLegend	3.5 µL
	CD285	624915	FITC	R&D Systems	3.5 µL
	CD303	201A	PE	BioLegend	3.5 µL
	CD161	L243	BV785	BioLegend	3.5 µL

Cells were incubated for 20 minutes at room temperature in the absence of light. 150 µL wash buffer was added to each well and the plates centrifuged at 330 g for 10 minutes. Cells were washed once more with wash buffer, resuspended in 200 µL wash buffer and transferred into Falcon 5 mL round bottom polystyrene test tubes. Tubes were stored in the absence of light at 4°C until acquisition.

2.5.9. Antigens used in in vitro assays

Various antigens were used to stimulate cell *ex vivo* in order to probe immunological function.

Table 2.14. *Ex vivo* antigen preparation

Study reference	Description	Manufacturer/Supplier	Preparation of stimuli
Chapter 3	Complete medium	-	100 µL complete medium
	Heat-killed Ty21a (Vivotif)	Crucel	100 µL complete medium containing 1×10^6 CFU heat-killed Ty21a
	Subunit Influenza (Influvac)	Abbott Laboratories	100 µL complete medium containing 45 ng hemagglutinin and neuraminidase from, in equal quantities, a A/Brisbane/59/2007 H1N1-like strain, a A/Brisbane/10/2007 H3N2-like strain and a B/Brisbane/60/2008-like strain
	Staphylococcal enterotoxin B (SEB)	Sigma	100 µL complete medium containing 100 ng SEB
Chapter 4	Complete medium	-	100 µL complete medium
	Heat-killed Ty21a	Crucel	100 µL complete medium containing 1×10^6 CFU heat-killed Ty21a
	Flagella (FliC)	Gift from Prof A. F. Cunningham	100 µL complete medium containing 5 µg FliC protein
	SEB	Sigma	100 µL complete medium containing 100 ng SEB
Chapter 5	Complete medium	-	100 µL complete medium
	Heat-killed Ty21a	Crucel	100 µL complete medium containing 2×10^6 CFU heat-killed Ty21a
	Heat-killed *Candida albicans*	Gift from Prof M. G. Netea	100 µL complete medium containing 2×10^6 CFU heat-killed C. albicans
	Purified protein derivative from *M. tuberculosis* (TB PPD)	Statens Serum Institut	100 µL complete medium containing 5 µg TB PPD
	Tetanus toxoid	Sigma	100 µL complete medium containing 20 µg tetanus toxoid
	Split Viron Influenza	Sanofi Pasteur	100 µL complete medium containing 180 ng hemagglutinin from, in equal quantities, a A/California/7/2009 H1N1-like strain, a A/Texas/50/20122 H3N2-like strain and a B/Massachusetts/2/2012-like strain

2.5.10. Ex vivo cell stimulation and staining

Seeded cells were stimulated with antigens listed in Table 2.14. Following the addition of antigen, cells were incubated at 37°C in 5% CO_2 for two hours. Cells were then treated with protein transport inhibitors resulting in the accumulation of protein within the Golgi complex for detection by flow cytometry (Table 2.15).

Table 2.15. Protein transport inhibitors

Study reference	Name	Description	Manufacturer	Per test
Chapter 3	BD GolgiPlug	Contains brefeldin A	BD Biosciences	1 µL
Chapter 4	BD GolgiPlug	Contains brefeldin A	BD Biosciences	1 µL
	BD GolgiStop	Contains monensin	BD Biosciences	1 µL
Chapter 5	BD GolgiPlug	Contains brefeldin A	BD Biosciences	1 µL
	BD GolgiStop	Contains monensin	BD Biosciences	1 µL

Cells were then incubated at 37°C in 5% CO_2 for 16 hours. Plates were centrifuged at 330 g for 10 minutes, the supernatant was removed and the cells washed with 200 µL wash buffer. Cells were resuspened in 50 µL wash buffer containing viability dye:

Table 2.16. Viability dyes for analysis of intracellular cytokine production

Study reference	Name	Description	Manufacturer	Per test
Chapter 3	LIVE/DEAD	Pacific Blue	LifeTechnologies	1 µL
Chapter 4	LIVE/DEAD	Pacific Blue	LifeTechnologies	0.02 µL
Chapter 5	LIVE/DEAD	Pacific Orange	LifeTechnologies	0.02 µL

Cells were incubated for 20 minutes at room temperature in the absence of light. 150 µL wash buffer was added to each well and the plates centrifuged at 330 g for 10 minutes. Cells were resuspened in 50 µL wash buffer containing the following antibodies:

Table 2.17. Surface antibody panel for identification of cell subsets

Study reference	Target	Clone	Fluorophore	Manufacturer	Per test
Chapter 3	CD3	HIT3a	APC	BD Biosciences	10 µL
	CD4	SK3	APC/Cy7	BD Biosciences	5 µL
	CD8	RPA-T8s	PE/Cy7	BD Biosciences	1 µL
	Integrinβ_7	FIB504	PE/Cy5	BD Biosciences	2 µL
	CD14	M5E2	HorizonV450	BD Biosciences	2 µL
	CD19	HIB19	HorizonV450	BD Biosciences	2 µL
Chapter 4	CD3	OCT3	BV/510	BioLegend	1 µL
	CD4	SK3	PE/Cy7	BD Biosciences	1 µL
	CD8	SK1	APC/H7	BD Biosciences	1 µL
	CD14	MϕP9	HorizonV450	BD Biosciences	1 µL
	CD19	HIB19	HorizonV450	BD Biosciences	1 µL
Chapter 5	CD3	SK7	APC/H7	BD Biosciences	1 µL
	CD4	SK3	PerCP/Cy5.5	BioLegend	1 µL
	CD8	SK1	BV/650	BioLegend	1 µL
	CD14	MϕP9	APC/H7	BD Biosciences	1 µL
	CD20	2H7	Pacific Blue	BioLegend	1 µL
	CD161	HP-3G10	BV/785	BioLegend	1 µL
	TCRγδ	11F2	PE/Cy7	BD Biosciences	1 µL
	Vα7.2	3C10	PE/Dazzle594	BioLegend	1 µL

Cells were incubated for 20 minutes at room temperature in the absence of light. 150 µL wash buffer was added to each well and the plates centrifuged at 330 g for 10 minutes. Cells were resuspened in 100 µL Cytofix/Cytoperm and incubated for 20 minutes at room temperature in the absence of light. 100 µL Perm/Wash was added to each well and the plates centrifuged at 330 g for 10 minutes. Cells were resuspended in 50 µL Perm/Wash containing the following antibodies:

Table 2.18. Intracellular antibody panel for identification of cell subsets

Study reference	Target	Clone	Fluorophore	Manufacturer	Volume
Chapter 3	IFN-γ	B27	AF700	BD Biosciences	1 µL
	TNF-α	MAb11	AF488	BD Biosciences	2.5 µL
	IL-2	MQ1-17H12	PE	BD Biosciences	10 µL
Chapter 4	CD69	FN50	PE/CF594	BD Biosciences	2 µL
	MIP-1β	24006	APC	R&D Systems	2 µL
	IFN-γ	B27	AF700	BD Biosciences	2 µL
	TNF-α	MAb11	AF488	BD Biosciences	2 µL
	IL-2	MQ1-17H12	PE	BD Biosciences	2 µL
	IL-17A	eBio64DEC17	PerCP/Cy5.5	eBioscience	2 µL
Chapter 5	IFN-γ	4S.B3	PE	BioLegend	2 µL
	TNF-α	MAb11	FITC	BioLegend	2 µL
	IL-17A	N49-653	AF700	BD Biosciences	2 µL
	IL-4	MP4-25D2	BV711	BD Biosciences	2 µL
	TGF-β	TW4-6H10	APC	BioLegend	2 µL

Cells were incubated for 30 minutes at room temperature in the absence of light. 150 µL Perm/Wash was added to each well and the plates centrifuged at 330 g for 10 minutes. Cells were washed once more with Perm/Wash, resuspended in 200 µL wash buffer and transferred into Falcon 5 mL round bottom polystyrene test tubes. Tubes were stored in the absence of light at 4°C until acquisition.

2.5.11. Flow cytometric acquisition and analyses

Flow cytometric data were acquired using either a LSR II flow cytometer (Chapter 3 and Chapter 4; BD Biosciences) or a FACSAria III (Chapter 5; BD Biosciences). Standard procedures were used to maintain both machines and quality control was performed daily. Compensation matrices were created using compensation beads (BD Biosciences). Analysis were performed using FlowJo version 7.6.5 (Chapter 3 and Chapter 4; Treestar Inc.) and FlowJo version 10.2 (Chapter 5; Treestar Inc.).

2.5.12. LPS ELISA

Plate coating

100 µL of carbonate-bicarbonate buffer containing 50 ng *S.* Typhi LPS was added to each well of 96 well flat-bottom microtitre plate. Plates were sealed and incubated at 4°C overnight.

Plate blocking

Plates were washed three times with PBS-tween and 100 µL 1.0% BSA was added to each well. Plates were sealed and incubated at room temperature for two hours.

Preparation and addition of sera

A standard was created using serum obtained from a patient previously diagnosed with typhoid (IgG; 1:450 – 1:328,050 and IgA; 1:50 – 1:36,450). Volunteer samples were diluted 1:3 four times across a range selected for optimum comparison against the standard (IgG; 1:150 – 1:4,050 and IgA; 1:50 – 1:1,350).

Once samples had been prepared, plates were washed three times with PBS-tween and 200 µL of each sample was added in duplicate. Plates were sealed and incubated at 4°C overnight.

Detection antibody

Plates were washed three times with PBS-tween.

- For the detection of IgG, a 1:4,000 dilution of anti-human-IgG was created using 0.1% BSA and 100 µL added to each well. Plates were sealed and incubated at room temperate for two hours.

- For the detection of IgA, 1:4,000 dilution of anti-human-IgA was created using 0.1% BSA and 100 µL added to each well. Plates were sealed and incubated at room temperate for two hours. Plates were washed three times with PBS-tween. A 1:2,000 dilution of streptavidin:alkaline phosphatase was created using 0.1%

BSA and 100 µL added to each well. Plates were sealed and incubated at room temperate for one hour.

Development and analyses

Plates were washed three times with PBS-tween and 100 µL of *p*-nitrophenyl phosphate was added to each well. Plates were incubated at room temperate for a fixed period (IgG; 90 minutes and IgA; 30 minutes) and the optical density of each well measured at 405 nm using a FLUOstar Omega ELISA microplate reader (BMG Labtech). The average blank corrected value was calculated for each sample and the data analysed using Omega Analysis (BMG Labtech).

2.5.13. Influenza ELISA

Plate coating

100 µL of carbonate-bicarbonate buffer containing 25 ng influenza hemagglutinin and neuraminidase antigens (Influvac containing, in equal quantities, a A/Brisbane/59/2007 H1N1-like strain, a A/Brisbane/10/2007 H3N2-like strain and a B/Brisbane/60/2008-like strain) to each well of 96 well flat-bottom microtitre plate (NUNC; 442404). Plates were sealed and incubated at 4°C overnight.

Plate blocking

Plates were washed three times with PBS-tween and 100 µL 1.0% BSA was added to each well. Plates were sealed and incubated at room temperature for two hours.

Preparation and addition of sera

A standard was created using serum obtained from a patient previously diagnosed with typhoid (IgG; 1:450 – 1:328,050 and IgA; 1:50 – 1:36,450). Volunteer samples were diluted 1:3 four times across a range selected for optimum comparison against the standard (IgG; 1: 4,050 – 1:109,350 and IgA; 1:150 – 1:4,050).

Once samples had been prepared, plates were washed three times with PBS-tween and 200 µL of each sample was added in duplicate. Plates were sealed and incubated at 4°C overnight.

Detection antibody

Plates were washed three times with PBS-tween.

- For the detection of IgG, a 1:4,000 dilution of anti-human-IgG was created using 0.1% BSA and 100 µL added to each well. Plates were sealed and incubated at room temperate for two hours.

- For the detection of IgA, 1:4,000 dilution of anti-human-IgA was created using 0.1% BSA and 100 µL added to each well. Plates were sealed and incubated at room temperate for two hours. Plates were washed three times with PBS-tween. A 1:2,000 dilution of streptavidin:alkaline phosphatase was created using 0.1% BSA and 100 µL added to each well. Plates were sealed and incubated at room temperate for one hour.

Development and analyses

Plates were washed three times with PBS-tween and 100 µL of *p*-nitrophenyl phosphate was added to each well. Plates were incubated at room temperate for a fixed period (IgG; 90 minutes and IgA; 30 minutes) and the optical density of each well measured at 405 nm using a FLUOstar Omega ELISA microplate reader (BMG Labtech). The average blank corrected value was calculated for each sample and the data analysed using Omega Analysis (BMG Labtech).

2.6. Sample acquisition

Chapter 3

Samples were acquired from vaccinated volunteers and control group volunteers at baseline (Day 0) and at day 18 – full details of sampling can be found in Figure 2.1. Samples were

acquired at day 18 since data suggested that, following an early increase in the frequency of antigen-specific T cells in peripheral blood at 2 to 4 days, a reduction was observed from 7 days up to between 28 and 56 days (Salerno-Goncalves et al., 2010). It was, therefore, hypothesised that during this period peripheral T cells traffic to the gut. The day 18 time point allowed us to efficiently utilise the endoscopy suite and sat within this period of peripheral decline.

Chapter 4

Volunteers were recalled as soon as possible once ethical approval had been granted for this study – full details of sampling can be found in Figure 2.2. Control group volunteers were recruited to facilitate unpaired comparison.

Chapter 5

Samples were acquired from vaccinated volunteers and control group volunteers at baseline (Day 0), 14 days, 3 months and 6 months – full details of sampling can be found in Figure 2.3. Samples were acquired at these time points since changes in monocyte phenotype and cytokine production had previously been observed at similar time points following vaccination with BCG (Kleinnijenhuis et al., 2014).

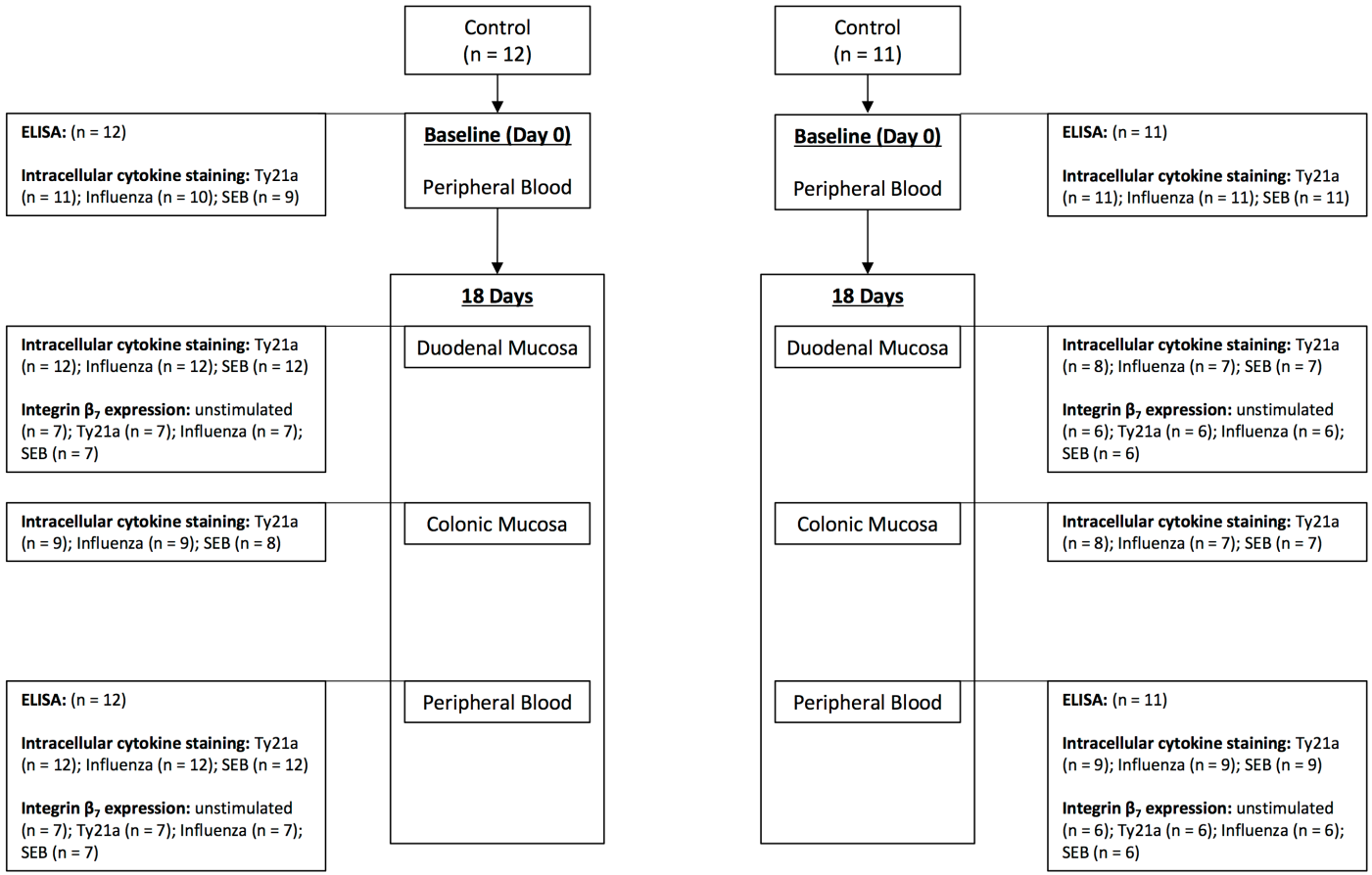

Figure 2.1. Consort flow diagram showing numbers of vaccinated and control volunteers from Chapter 3, with sampling information at each time point as well as experimental output

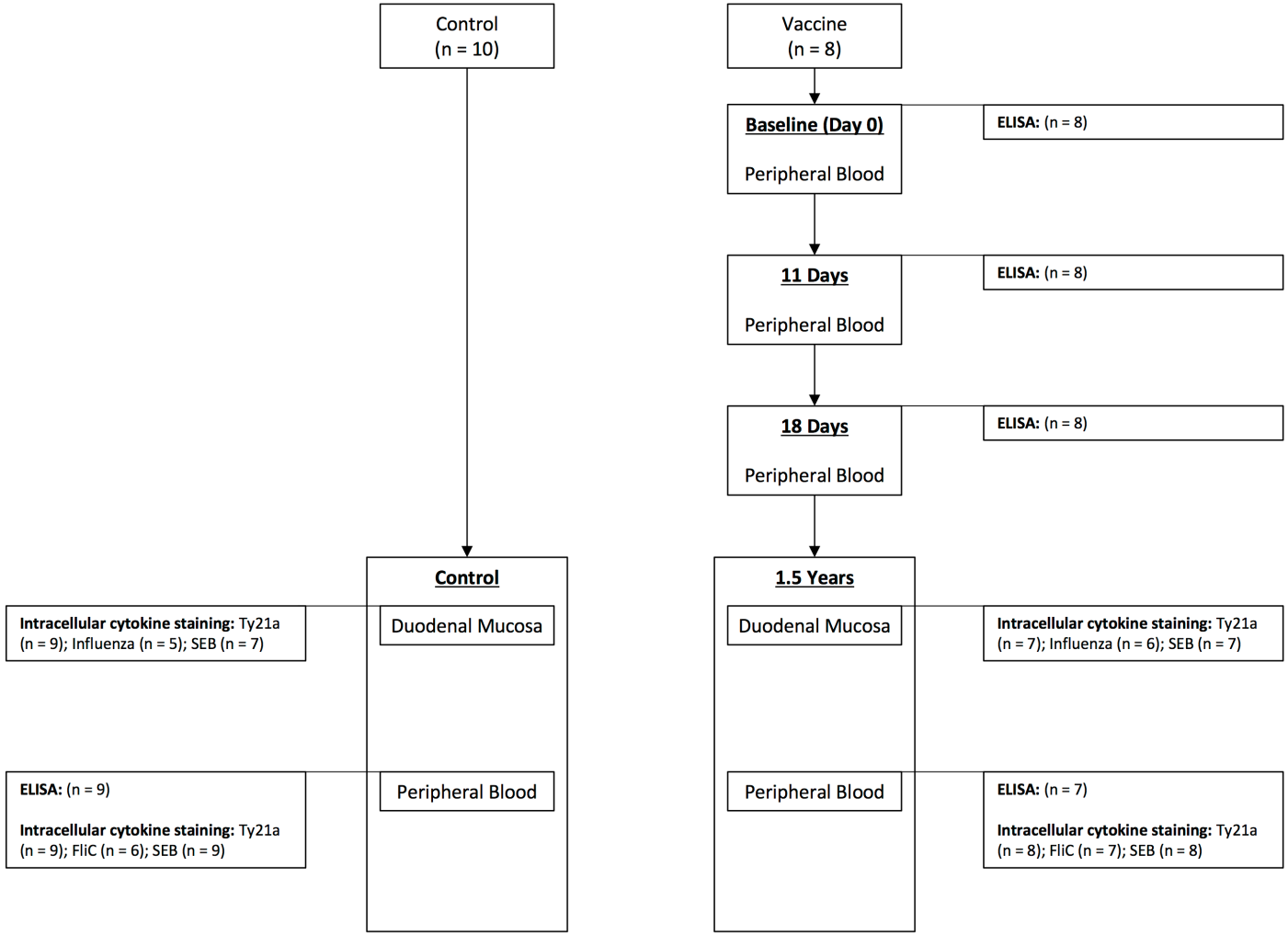

Figure 2.2. Consort flow diagram showing numbers of vaccinated and control volunteers from Chapter 4, with sampling information at each time point as well as experimental output

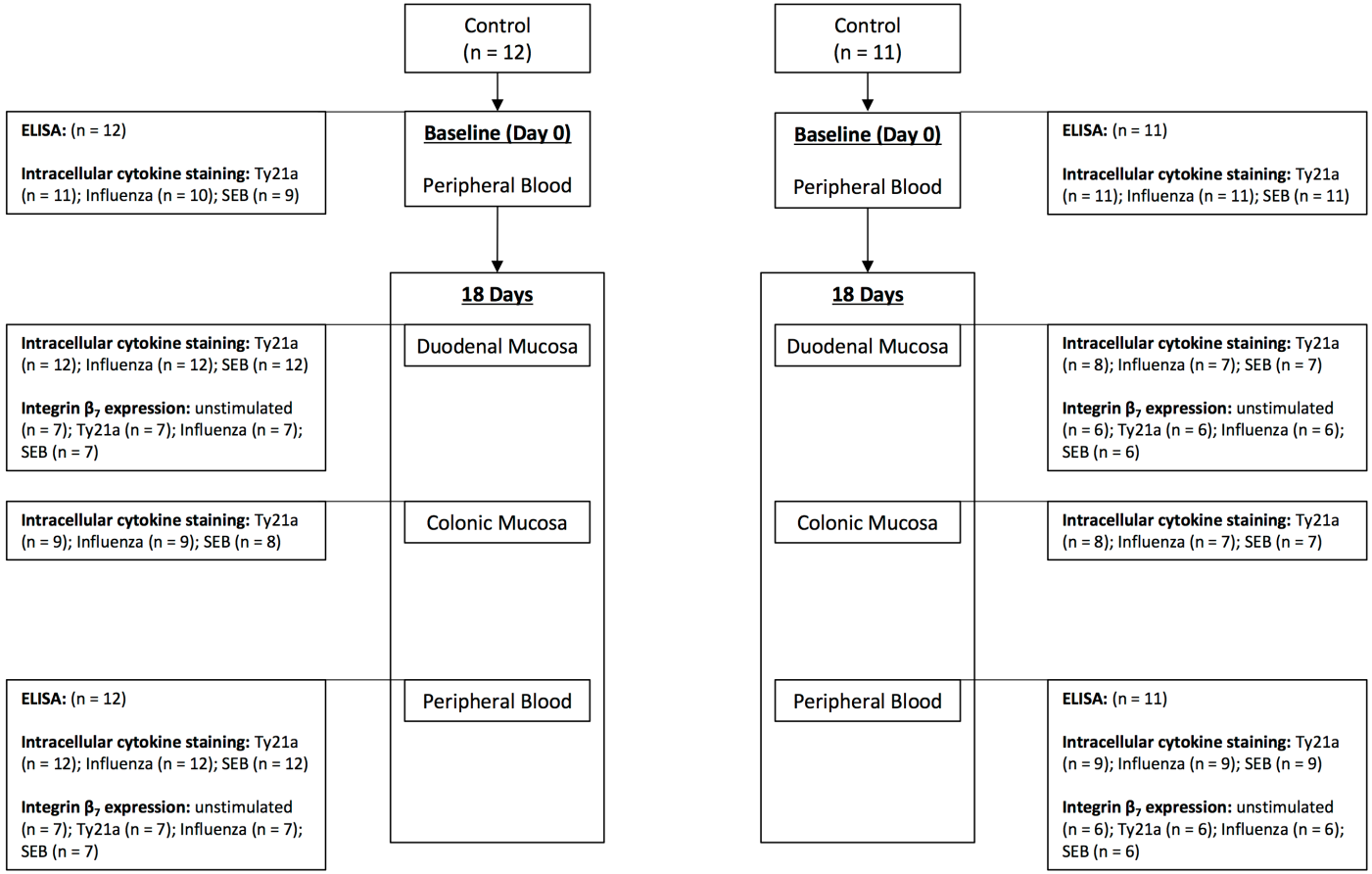

Figure 2.3. Consort flow diagram showing numbers of vaccinated and control volunteers from Chapter 5, with sampling information at each time point as well as experimental output

2.7. Data management and statistical analyses

Chapter 3

- Paired *t* tests were performed using logarithmically transformed data; *P* values are 2-tailed and considered significant at $P < .05$ Prism (Prism; GraphPad)
- Unpaired *t* tests were performed using untransformed data; *P* values are 2-tailed and considered significant at $P < .05$ Prism (Prism; GraphPad)
- Associations were measured using the Pearson correlation coefficient; *P* values are 2-tailed and considered significant at $P < .05$ (Prism; GraphPad)

Chapter 4

- Paired *t* tests were performed using logarithmically transformed bootstrapped data; data were considered significant if 95% confidence interval did not cross 0 (SPSS; IBM)
- Unpaired *t* tests were performed using untransformed bootstrapped data; data were considered significant if 95% confidence interval did not cross 0 (SPSS; IBM)

Chapter 5

- Paired *t* tests were performed using untransformed data; *P* values are 2-tailed and considered significant at $P < .05$ (Prism; GraphPad)
- Each volunteer was assigned a numerical value based on whether there was a reduction in output (-1; <80% of baseline), no change in output (0; between $\leq$80% and $\geq$120% of baseline), or increased output (+1; <120% of baseline) at T1, T2 and T3. Linear discriminant analysis of principal components (DAPC) was used to determine the capacity to model data to determine vaccine status (R; R Foundation for Statistical Computing)

CHAPTER 3: THE ADAPTIVE IMMUNE RESPONSE TO TY21A AT DAY 18

3.1. Introduction

Ty21a is able to induce humoral and cellular immune responses, both of which have been implicated in protection against disease. While opsonophagocytic immunoglobulin function (Wahid et al., 2014), cellular cytotoxicity, proliferation and cytokine production functionality have been assessed in peripheral blood following vaccination with Ty21a (Salerno-Goncalves et al., 2002, Lundin et al., 2002, Salerno-Goncalves et al., 2004, Salerno-Goncalves et al., 2005, Salerno-Goncalves et al., 2010, McArthur and Sztein, 2012), cellular immunity at the human intestinal mucosa has never been directly assessed. Numerous studies have demonstrated that cellular immune responses generated through vaccination with Ty21a are primed for mucosal homing (Kantele et al., 1997, Kantele et al., 1999b, Lundin et al., 2002), highlighting the importance of mucosal immunity in defence against disease. Furthermore, it has been demonstrated that the assessment of vaccine immunogenicity by peripheral

sampling alone provides an incomplete reflection of vaccine immunogenicity (Thome et al., 2014).

It has previously been observed in murine models that previously primed T cells of heterologous specificities are recruited to the lung during influenza virus infection (Chapman et al., 2005). Although this phenomenon has not been observed in humans, we hypothesised that vaccination with Ty21a could enhance T-cell responses to heterologous antigens at the mucosal surface by a similar mechanism.

Through the direct assessment of immunity at the intestinal mucosa, it may be possible to identify mechanisms involved in the induction of protective immunity, which may be manipulated to improve oral vaccine immunogenicity. Here, we have assessed cellular immunity in vaccinated volunteers and controls at the duodenal and colonic mucosa and in peripheral blood after 18 days. We have compared and correlated peripheral and mucosal cellular responses with peripheral humoral measures of vaccine efficacy, providing a unique insight into the relationship between human mucosal and peripheral immune defence.

3.1.1. Hypotheses

We hypothesised that:

- Responses at the intestinal mucosa would be stronger than those in peripheral blood
- Responses would be observed at the intestinal mucosa which were not apparent in peripheral blood

3.2. Methods

Full details of the methods used to produce the data which are presented within this chapter can be found in Chapter 2. Some data which are pertinent to this chapter are included for ease of reference.

3.2.1. Ethical approval and recruitment

Full details can be found in section 2.1. The following is a table of volunteer demographics:

Table 3.1. Chapter 3 volunteer demographics

Group	*n*	Gender (M:F)
Control	12	3:9
Vaccine	11	6:5

3.2.1. Vaccination

Full details can be found in section 2.3.

3.2.2. Clinical sampling

Full details of venesection and endoscopy can be found in sections 2.4.2 and 2.4.3, respectively.

3.2.3. Serum isolation

Full details can be found in section 2.5.4.

3.2.4. ELISA

Full details of ELISAs specific for LPS and influenza-virus can be found in sections 2.5.12 and 2.5.13, respectively.

3.2.5. PBMC isolation

Full details can be found in section 2.5.5.

3.2.6. MMC isolation

Full details can be found in section 2.5.7.

3.2.7. Ex vivo cell stimulation and staining

Full details of the antigens used for stimulation can be found in section 2.5.9. Antigens used in the chapter include:

Table 3.2. Antigens used for *in vitro* stimulation

Description	Preparation of stimuli
Negative control	100 µL complete medium
Heat-killed Ty21a (Vivotif)	100 µL complete medium containing 1×10^6 CFU heat-killed Ty21a
Subunit Influenza (Influvac)	100 µL complete medium containing 45 ng hemagglutinin and neuraminidase from, in equal quantities, a A/Brisbane/59/2007 H1N1-like strain, a A/Brisbane/10/2007 H3N2-like strain and a B/Brisbane/60/2008-like strain
SEB	100 µL complete medium containing 100 ng SEB

Full details of the stimulation and staining protocol can be found in section 2.5.10. Antibodies used in this chapter include:

Table 3.3. Dyes and antibodies used for analysis of intracellular cytokine production

	Target	Clone	Fluorophore	Manufacturer	Per test
Viability	Dead cells	N/A	Pacific Blue	LifeTechnologies	1 µL
Extracellular	CD3	HIT3a	APC	BD Biosciences	10 µL
	CD4	SK3	APC/Cy7	BD Biosciences	5 µL
	CD8	RPA-T8s	PE/Cy7	BD Biosciences	1 µL
	Integrinβ_7	FIB504	PE/Cy5	BD Biosciences	2 µL
	CD14	M5E2	HorizonV450	BD Biosciences	2 µL
	CD19	HIB19	HorizonV450	BD Biosciences	2 µL
Intracellular	IFN-γ	B27	AF700	BD Biosciences	1 µL
	TNF-α	MAb11	AF488	BD Biosciences	2.5 µL
	IL-2	MQ1-17H12	PE	BD Biosciences	10 µL

3.2.8. Flow cytometric acquisition and analyses

Full details can be found in section 2.5.11.

3.2.9. Data management and statistical analysis

Full details can be found in section 2.6.

3.3. Results

3.3.1. Serum immunoglobulin specificity

Ty21a-mediated protection is dependent upon the expression of LPS (Germanier and Fuer, 1975), and, in field trials, humoral responses to LPS were shown to correlate with vaccine efficacy (Levine et al., 1989). We compared levels of serum anti-LPS IgG and IgA prior to and following vaccination. We also measured levels of serum IgG and IgA specific to influenza virus, a common naturally encountered pathogen, to assess the impact of vaccination on humoral immunity to a heterologous pathogen. Influenza virus was selected since the majority, if not all, volunteers would have been exposed to this pathogen in the community.

While levels of anti-LPS serum IgG and IgA among unvaccinated volunteers were not different between day 18 and day 0, among the vaccinated levels of anti-LPS serum IgG were 3-fold higher and levels of levels of anti-LPS serum IgG were also 3 -fold higher at day 18 than at day 0 (P = .03 and P = .01; Figure 3.1A). Levels of anti-influenza serum IgG or IgA among the unvaccinated and the vaccinated volunteers were not different between day 18 and day 0 (Figure 3.1B).

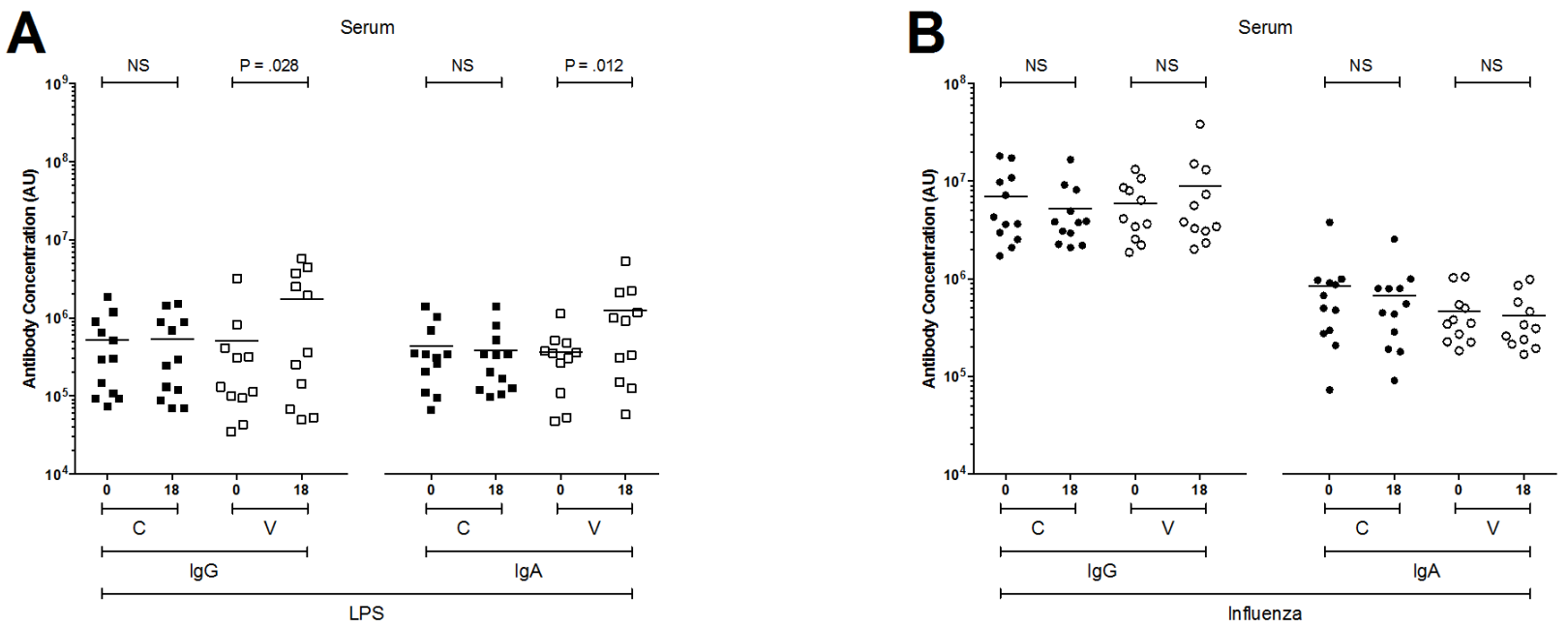

Figure 3.1. Levels of serum immunoglobulin (Ig)G and IgA to *Salmonella* Typhi lipopolysaccharide (LPS) and influenza virus

The levels of IgG and IgA specific to *Salmonella* Typhi LPS (A) and influenza virus (B) in serum, expressed in arbitrary units (AU). For control (C; closed squares and circles [LPS n = 12, Influenza n = 12]) and vaccinated (V; open squares and circles [LPS n = 11, Influenza n = 11]) volunteers, paired comparisons were made between day 0 and day 18 values. Horizontal bars represent mean values (paired *t* tests were performed using logarithmically transformed data). Abbreviation: ns, not significant.

3.3.2. Peripheral blood and gut mucosal cellular responses

We compared the frequency of Ty21a-responsive T cells in vaccinated volunteers and controls, at the duodenal and colonic mucosa and in peripheral blood. We also measured the frequency of influenza virus-responsive T cells. A combinatorial gating strategy was used to identify the proportion of $CD4^+$ and $CD8^+$ T cells positive for any combination of IFN-γ, TNF-α, and/or IL-2 (Figure 3.2). Cytokine production in non-stimulated samples (negative control) was minimal, did not differ between vaccinated and unvaccinated volunteers and was subsequently subtracted from other conditions. Cytokine production in SEB–stimulated samples (positive control) was high and did not differ between vaccinated and unvaccinated volunteers.

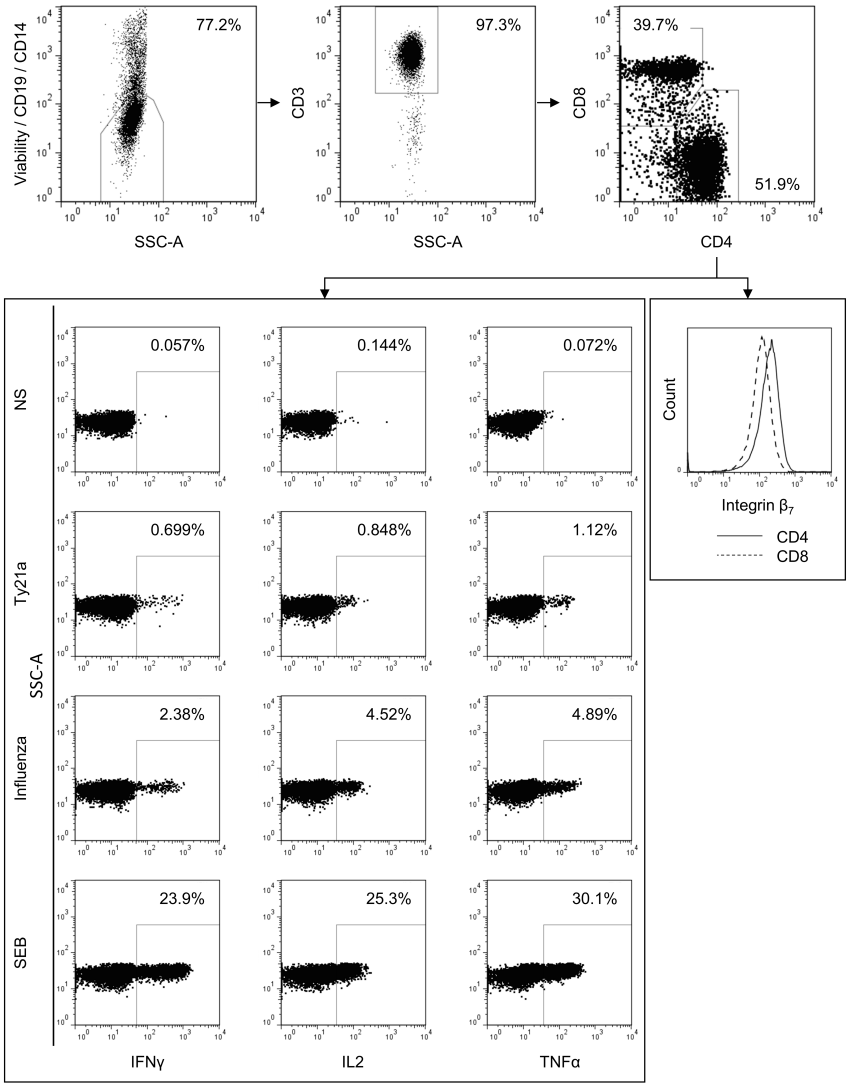

Figure 3.2 (A). Representative flow cytometric gating strategy for intracellular cytokine analysis

Dot plots are shown for cells isolated from the duodenal mucosa. Dead cells, B cells and monocytes were removed by staining for viability (LIVE/DEAD), CD19 and CD14 and gating on the negative population. T cells were identified according to the expression of CD3. T cells were classified according to the expression of CD4 and CD8 and the expression of interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and/or interleukin 2 (IL-2) assessed in non-stimulated and in live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a)-, influenza virus- and staphylococcal enterotoxin B (SEB)-stimulated samples. The expression intensity of integrin β_7 was also assessed.

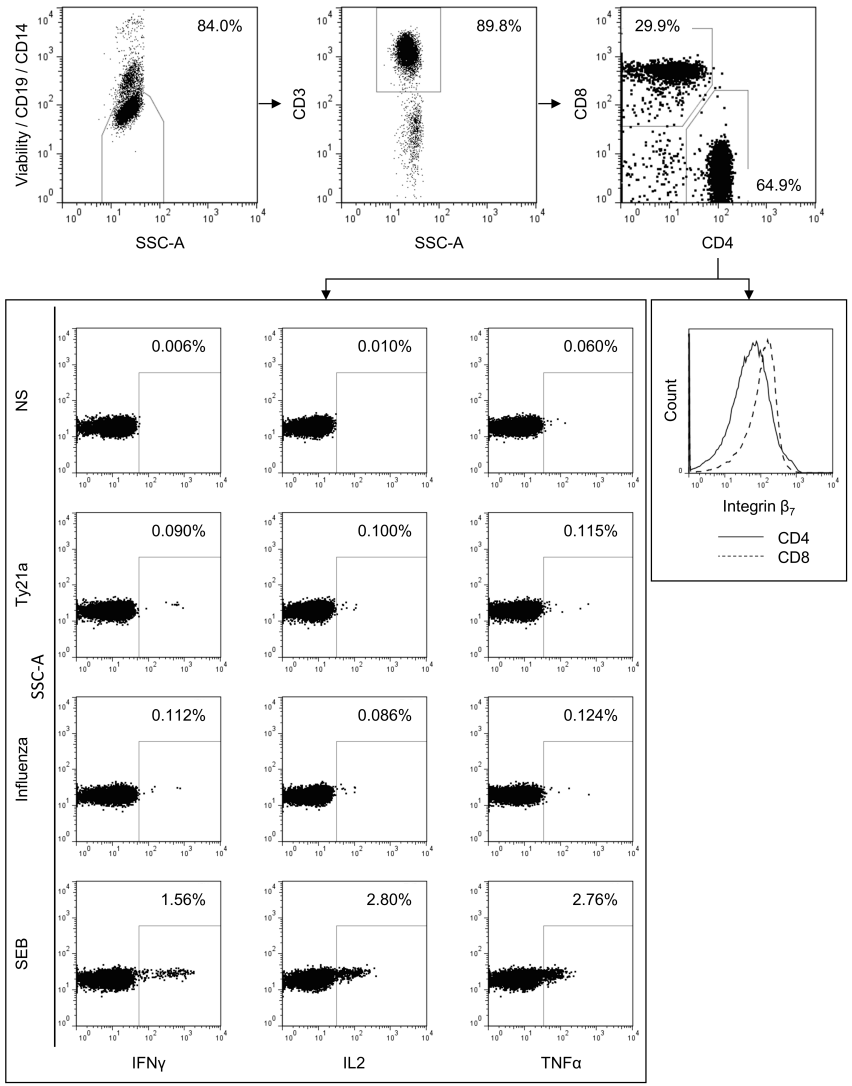

Figure 3.2 (B). Representative flow cytometric gating strategy for intracellular cytokine analysis

Dot plots are shown for cells isolated from peripheral blood. Dead cells, B cells and monocytes were removed by staining for viability (LIVE/DEAD), CD19 and CD14 and gating on the negative population. T cells were identified according to the expression of CD3. T cells were classified according to the expression of CD4 and CD8 and the expression of interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and/or interleukin 2 (IL-2) assessed in non-stimulated and in live-attenuated Salmonella Typhi strain Ty21a (Ty21a)-, influenza virus- and staphylococcal enterotoxin B (SEB)-stimulated samples. The expression intensity of integrin β7 was also assessed.

At day 0, in peripheral blood, the frequency of Ty21a-responsive and heterologous influenza virus-responsive $CD4^+$ and $CD8^+$ T cells in the vaccinated group was not different from the frequency in the unvaccinated control group (Figure 3.3). These data suggest that groups were well matched for prior exposure to Ty21a and influenza virus antigens and that any differences observed thereafter may be attributed to an effect of vaccination with Ty21a. Paired comparisons between day 0 and day 18 were not made in peripheral blood, as overnight fasting, required prior to endoscopy, is known to influence cytokine production in response to re-stimulation with bacterial and viral antigens (van den Brink et al., 2002).

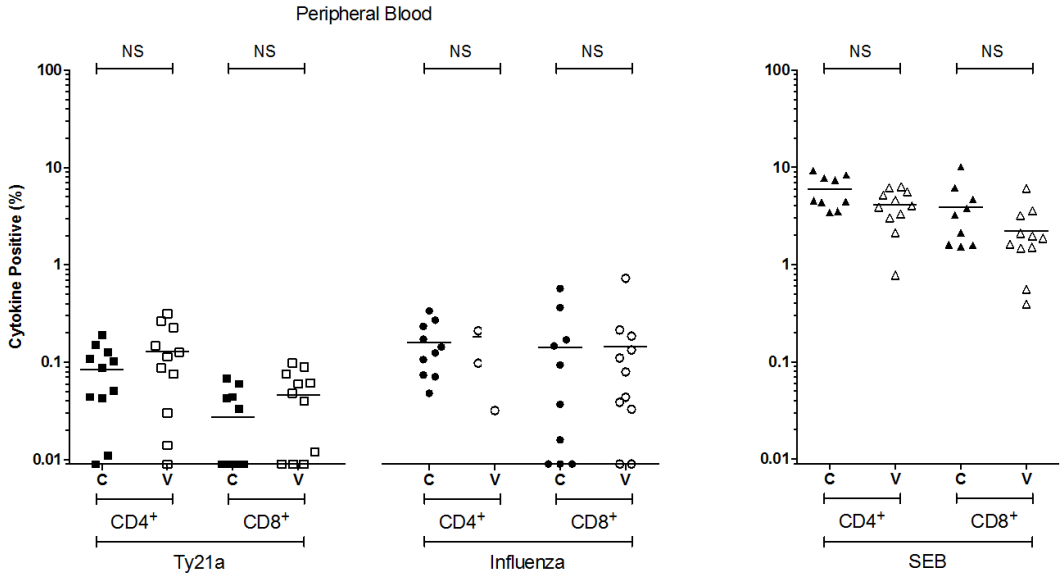

Figure 3.3. Antigen-specific cytokine-producing populations at day 0

The frequency of $CD4^+$ and $CD8^+$ live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a)-responsive and heterologous influenza virus-responsive populations expressing any combination of interferon γ, tumor necrosis factor α, and/or interleukin 2 above background. Staphylococcal enterotoxin B (SEB)-stimulated control data are also included. For control (C; closed squares, circles and triangles [Ty21a n = 11, Influenza n = 10, SEB n = 9]) and vaccinated (V; open squares, circles and triangles [Ty21a n = 11, Influenza n = 11, SEB n = 11]) volunteers, measurements were made in peripheral blood. Horizontal bars represent mean values (comparisons were made using unpaired *t* tests). Abbreviation: ns, not significant.

At day 18, at the duodenal mucosa, the frequency of Ty21a-responsive $CD4^+$ T cells was 3-fold higher and the frequency of Ty21a-responsive $CD8^+$ T cells 5-fold higher in vaccinated

volunteers, compared with unvaccinated volunteers (*P* = .007 and *P* = <.0001, respectively; Figure 3.4A). The frequency of heterologous influenza virus-responsive $CD4^+$ T cells was 5-fold higher and the frequency of heterologous influenza virus-responsive $CD8^+$ T cells 6-fold higher in vaccinated volunteers, compared with unvaccinated volunteers (*P* = .005 and *P* = .01, respectively; Figure 3.4A). At the colonic mucosa, there was no significant difference between the frequencies of Ty21a-responsive or heterologous influenza virus-responsive T cells in vaccinated volunteers, compared with unvaccinated volunteers (Figure 3.4B).

In peripheral blood, the frequency of Ty21a-responsive $CD4^+$ T cells was 4-fold higher in vaccinated volunteers, compared with unvaccinated volunteers (*P* = .019; Figure 3.4C). Vaccination did not influence the frequency of peripheral Ty21a-responsive $CD8^+$ T cells, nor the frequency of heterologous influenza virus-responsive $CD4^+$ or $CD8^+$ T cells at day 18.

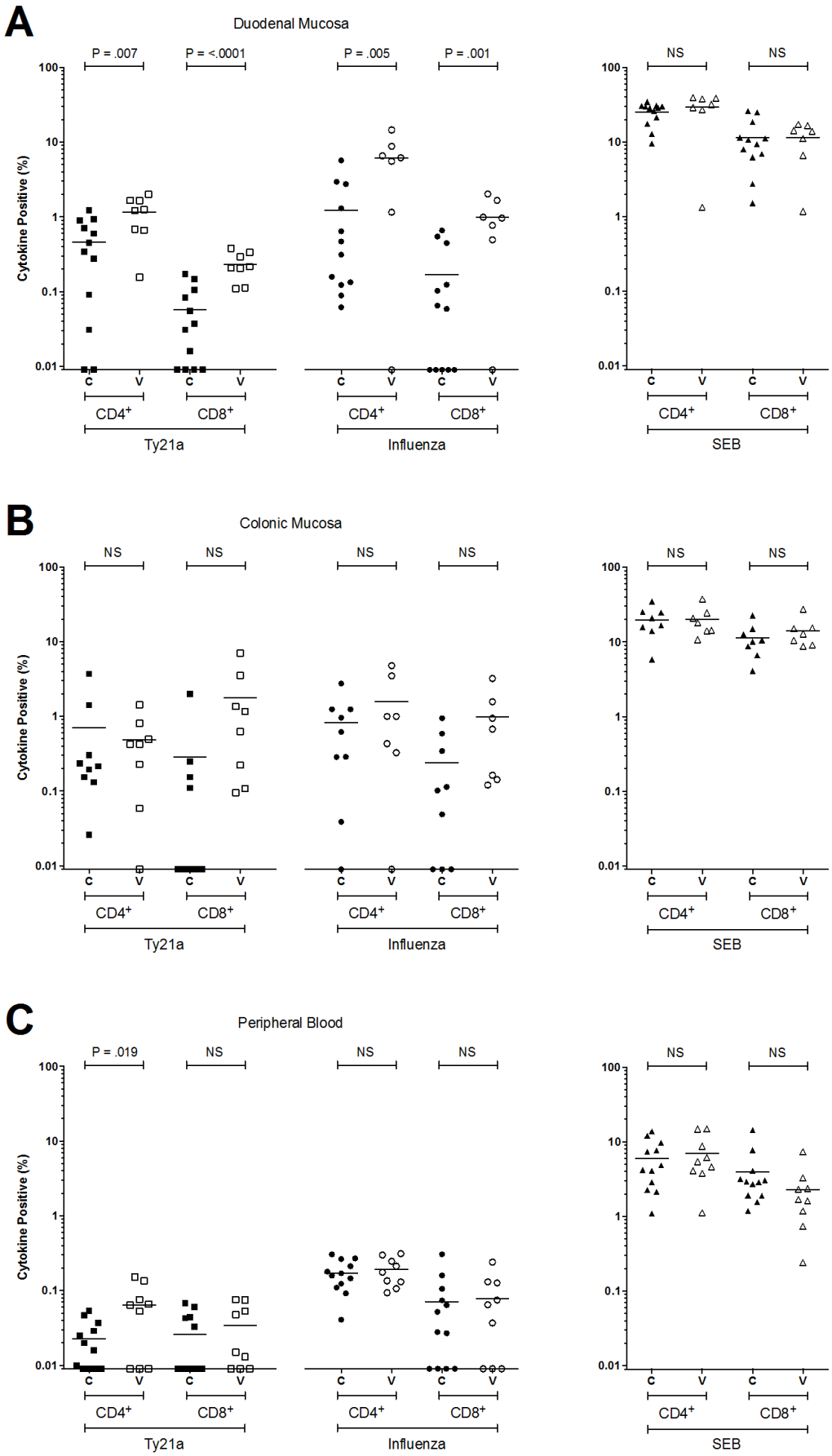
A
Duodenal Mucosa
P = .007
P = <.0001
P = .005
P = .001
NS
NS
Cytokine Positive (%)
100
10
1
0.1
0.01
C
V
CD4+
CD8+
Ty21a
Influenza
SEB
B
Colonic Mucosa
C
Peripheral Blood
P = .019

Figure 3.4. Antigen-specific cytokine-producing populations at day 18

The frequency of $CD4^+$ and $CD8^+$ live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a)-responsive and heterologous influenza virus–responsive populations expressing any combination of interferon γ, tumor necrosis factor α, and/or interleukin 2 above background. Staphylococcal enterotoxin B (SEB)-stimulated control data are also included. For control (C; closed squares, circles and triangles [duodenal mucosa; Ty21a n = 12, Influenza n = 12, SEB n = 12] [colonic mucosa; Ty21a n = 9, Influenza n = 9, SEB n = 8] [peripheral blood; Ty21a n = 12, Influenza n = 12, SEB n = 12]) and vaccinated (V; open squares, circles and triangles [duodenal mucosa; Ty21a n = 8, Influenza n = 7, SEB n = 7] [colonic mucosa; Ty21a n = 8, Influenza n = 7, SEB n = 7] [peripheral blood; Ty21a n = 9, Influenza n = 9, SEB n = 9]) volunteers, measurements were made at the duodenal mucosa (A), the colonic mucosa (B) and in peripheral blood (C). Horizontal bars represent mean values (comparisons were made using unpaired *t* tests). Abbreviation: ns, not significant.

3.3.3. Characteristics and functionality of cellular responses

Polyfunctional T cells, defined as cells that express multiple cytokines simultaneously, have been shown to correlate with vaccine-mediated protection against other intracellular infections (Darrah et al., 2007, Kannanganat et al., 2007). We compared the cytokine expression profile of vaccinated volunteers with that of unvaccinated volunteers to assess the functionality of responses to Ty21a and influenza virus antigens.

At the duodenal mucosa, the $CD4^+$ T-cell response to Ty21a was functionally heterogeneous, with the frequency of cells expressing one, two, or three cytokines being higher in vaccinated volunteers, compared with unvaccinated volunteers (P=.006, *P* = .018 and *P* = .031, respectively; Figure 3.5A). The duodenal $CD8^+$ T-cell response to Ty21a antigens was, in contrast, largely attributable to cells expressing just one cytokine (*P* = .0004; Figure 3.5A). Duodenal $CD4^+$ and $CD8^+$ T-cell responses to influenza virus antigens were also functionally heterogeneous, with the frequency of cells expressing one, two, or three cytokines being higher in vaccinated volunteers, compared with unvaccinated volunteers ($CD4^+$ T cells, *P* = .008, *P* = .005 and *P* = .005, respectively; $CD8^+$ T cells, *P* = .0008, *P* = .010 and *P* = .011; Figure 3.5B).

In peripheral blood, the $CD4^+$ T-cell response to Ty21a antigens was functionally heterogeneous, with the frequency of cells expressing one, two, or three cytokines being higher in vaccinated volunteers, compared with unvaccinated volunteers (P = .026, P = .028 and P = .008, respectively; Figure 3.5C). Analysis of expression profiles by individual cytokine demonstrated that responses to Ty21a and influenza virus antigens were attributable to cells producing combinations of IFN-γ, TNF-α and IL-2 but that no one cytokine predominated (Figure 3.6).

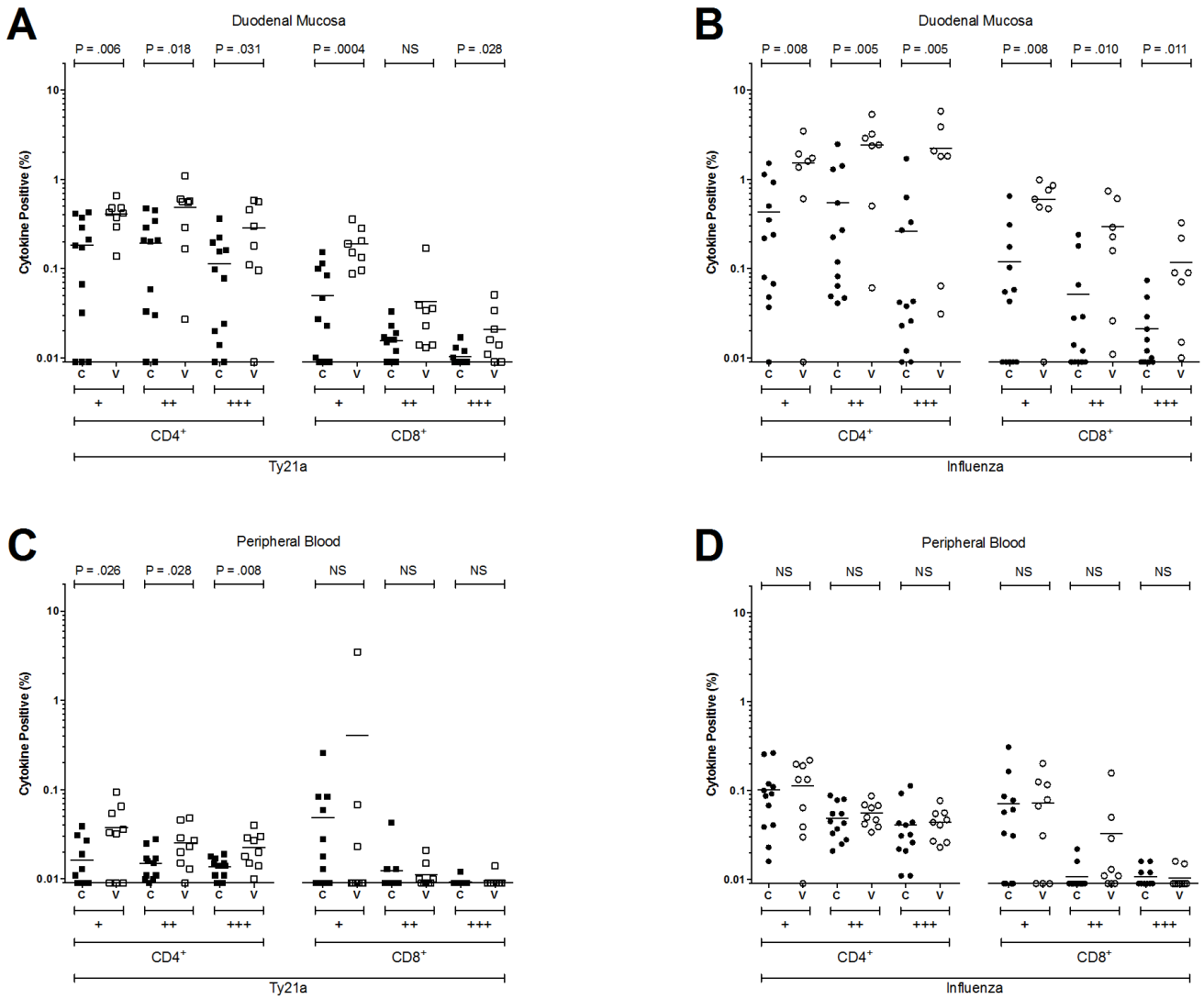

Figure 3.5. Combinations of antigen-specific cytokine production at day 18

The frequency of $CD4^+$ and $CD8^+$ live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a)-responsive and heterologous influenza virus-responsive populations expressing one (+), two (++), or three (+++) cytokines (interferon γ, tumor necrosis factor α, and/or interleukin 2) above background. For control (C; closed squares and circles [duodenal mucosa; Ty21a n = 12, Influenza n = 12] [peripheral blood; Ty21a n = 12, Influenza n = 12]) and vaccinated (V; open squares and circles [duodenal mucosa; Ty21a n = 8, Influenza n = 7] [peripheral blood; Ty21a n = 9, Influenza n = 9]) volunteers, measurements were made at the duodenal mucosa (A and B) and in peripheral blood (C and D). Horizontal bars represent mean values (comparisons were made using unpaired t tests). Abbreviation: ns, not significant.

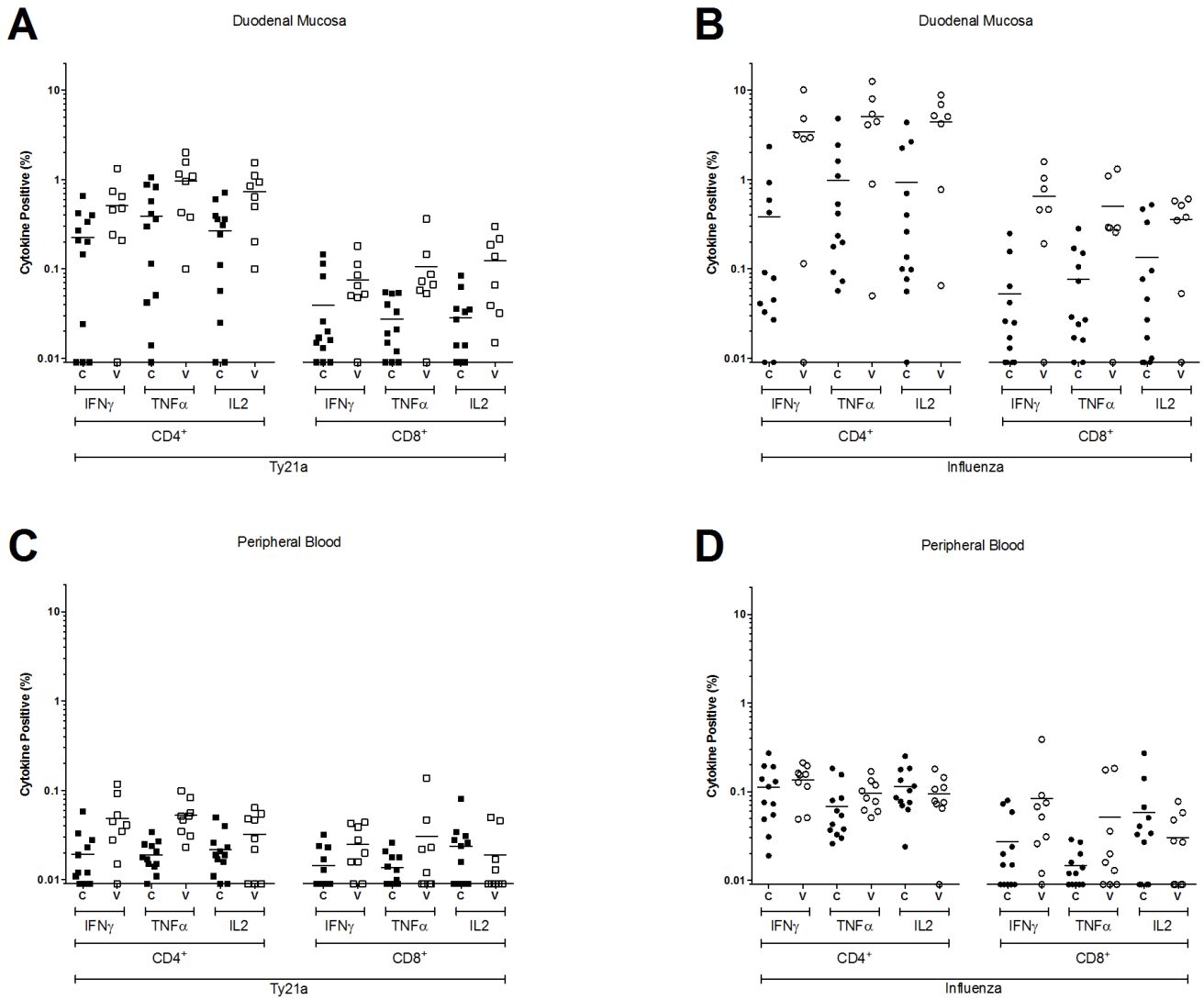

Figure 3.6. Antigen-specific production of individual cytokines at day 18

The frequency of $CD4^+$ and $CD8^+$ live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a)-responsive and heterologous influenza virus-responsive populations expressing IFN-γ, TNF-α, or IL-2 above background. For control (C; closed squares and circles [duodenal mucosa; Ty21a n = 12, Influenza n = 12] [peripheral blood; Ty21a n = 12, Influenza n = 12]) and vaccinated (V; open squares and circles [duodenal mucosa; Ty21a n = 8, Influenza n = 7] [peripheral blood; Ty21a n = 9, Influenza n = 9]) volunteers, measurements were made at the duodenal mucosa (A and B) and in peripheral blood (C and D). Horizontal bars represent mean values.

3.3.4. Correlations between cellular populations at the duodenal mucosa

We explored the relationship between the generation of Ty21a-responsive and heterologous influenza virus-responsive T cells at the duodenal mucosa. The frequencies of Ty21a-responsive $CD4^+$ and $CD8^+$ T cells were correlated (r^2 = 0.537 and *P* = .015; Table 3.4), as were the frequencies of heterologous influenza virus-responsive $CD4^+$ and $CD8^+$ T cells (r^2 = 0.930 and *P* = <.0001; Table 1). The frequencies of Ty21a-responsive $CD4^+$ and $CD8^+$ T cells also correlated robustly with the frequencies of corresponding influenza virus-responsive T cells ($CD4^+$ T cells, r^2 = 0.74 and *P* = .0003; $CD8^+$ T cells, r^2 = 0.677 and *P* = .001; Table 3.4).

3.3.5. Correlations between peripheral and mucosal immunity

We explored the relationship between the generation of peripheral and duodenal immune responses. The frequency of peripheral Ty21a-responsive $CD4^+$ T cells was modestly correlated with the frequency of duodenal Ty21a-responsive $CD4^+$ T cells (r^2 = 0.530 and *P* = .024; Table 3.4) but not with the frequency of duodenal Ty21a-responsive $CD8^+$ T cells or with the frequency of heterologous influenza virus-responsive $CD4^+$ and $CD8^+$ T cells. Levels of serum anti-LPS IgG and IgA correlated with the frequency of duodenal Ty21a-responsive $CD4^+$ T cells (IgG, r^2 = 0.542 and *P* = .014; IgA, r^2 = 0.710 and *P* = .0004; Table 3.4). Levels of serum IgA specific to LPS also correlated with the frequency of duodenal heterologous influenza virus-responsive $CD4^+$ and $CD8^+$ T cells ($CD4^+$ T cells, r^2 = 0.512 and *P* = .025; $CD8^+$ T cells, r^2 = 0.585 and *P* = .008; Table 3.4). Levels of anti-influenza virus IgG and IgA did not, however, correlate with either the frequency of Ty21a-responsive or heterologous influenza virus-responsive T cells at the duodenal mucosa in either subset.

Table 3.4. Pearson correlation analysis of cellular and humoral immune responses

			DUODENAL MUCOSA				PERIPHERAL BLOOD				SERUM			
			Ty21a		Influenza		Ty21a		Influenza		*S.* Typhi LPS		Influenza	
			$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$	IgG	IgA	IgG	IgA
DUODENAL MUCOSA	Ty21a	$CD4^+$	1	0.537 *	0.741 **	0.724 **	0.530 *	0.319 NS	-0.102 NS	-0.223 NS	0.542 *	0.710 *	0.160 NS	0.008 NS
		$CD8^+$		1	0.587 **	0.677 **	0.395 NS	0.126 NS	-0.117 NS	-0.232 NS	0.315 NS	0.396 NS	0.177 NS	-0.348 NS
	Influenza	$CD4^+$			1	0.929 **	0.327 NS	0.299 NS	-0.025 NS	-0.122 NS	0.392 NS	0.512 *	-0.245 NS	-0.228 NS
		$CD8^+$				1	0.394 NS	0.515 NS	0.099 NS	0.080 NS	0.392 NS	0.585 **	-0.149 NS	-0.300 NS

Abbreviation: ns, non-significant; *P <.05; **P <.001

3.3.6. Cellular potential for mucosal homing and residence

Integrin β_7 plays a prominent role in mucosal cellular immune defence; $\alpha_4\beta_7$ facilitates peripheral trafficking to the intestinal mucosa and $\alpha_\varepsilon\beta_7$ helps maintain resident intraepithelial lymphocyte populations (Berlin et al., 1993, Cepek et al., 1994). Since duodenal $CD4^+$ and $CD8^+$ T-cell responses to Ty21a and influenza virus antigens were closely correlated, we assessed the cellular capacity for mucosal homing and residence, which is a potential mechanism by which Ty21a vaccination may have influenced trafficking to the duodenal mucosa. We assessed integrin β_7 expression intensity, a component of both $\alpha_4\beta_7$ in peripheral blood and $\alpha\varepsilon\beta_7$ at the duodenal mucosa. A combinatorial gating strategy was used to determine the level of integrin β_7 expression among $CD4^+$ and $CD8^+$ T-cell populations (Figure 3.2).

At the duodenal mucosa, integrin β_7 expression was more intense among the total $CD4^+$ T-cell population than among the total $CD8^+$ T-cell population (*P* = <.0001; Figure 3.7A). Integrin β_7 expression intensity among Ty21a-responsive and influenza virus-responsive $CD4^+$ T-cell subpopulations was not different from that among the total mucosal $CD4^+$ T-cell population. In contrast, integrin β_7 expression intensity among Ty21a-responsive and influenza virus-responsive $CD8^+$ T-cell subpopulations was greater than that among the total mucosal $CD8^+$ T-cell population (*P* = .0002 and *P* = <.0001, respectively; Figure 3.7A).

In contrast with observations made at the duodenal mucosa, integrin β_7 expression was more intense among the total $CD8^+$ T-cell population than among the total $CD4^+$ T-cell population in peripheral blood (*P* = .0005; Figure 3.7B). While integrin β_7 expression intensity among the Ty21a-responsive $CD4^+$ T-cell subpopulation was greater than that among the total $CD4^+$ T-cell population (*P* = .002; Figure 3.7B), expression intensity among the influenza virus-responsive $CD4^+$ T-cell subpopulation was not different from that among the total $CD4^+$ T-cell population. Integrin β_7 expression intensity among the Ty21a-responsive and

heterologous influenza virus-responsive $CD8^+$ T-cell subpopulations was greater than that among the total $CD8^+$ T-cell population (*P* = .004 and *P* = .041, respectively; Figure 3.7B).

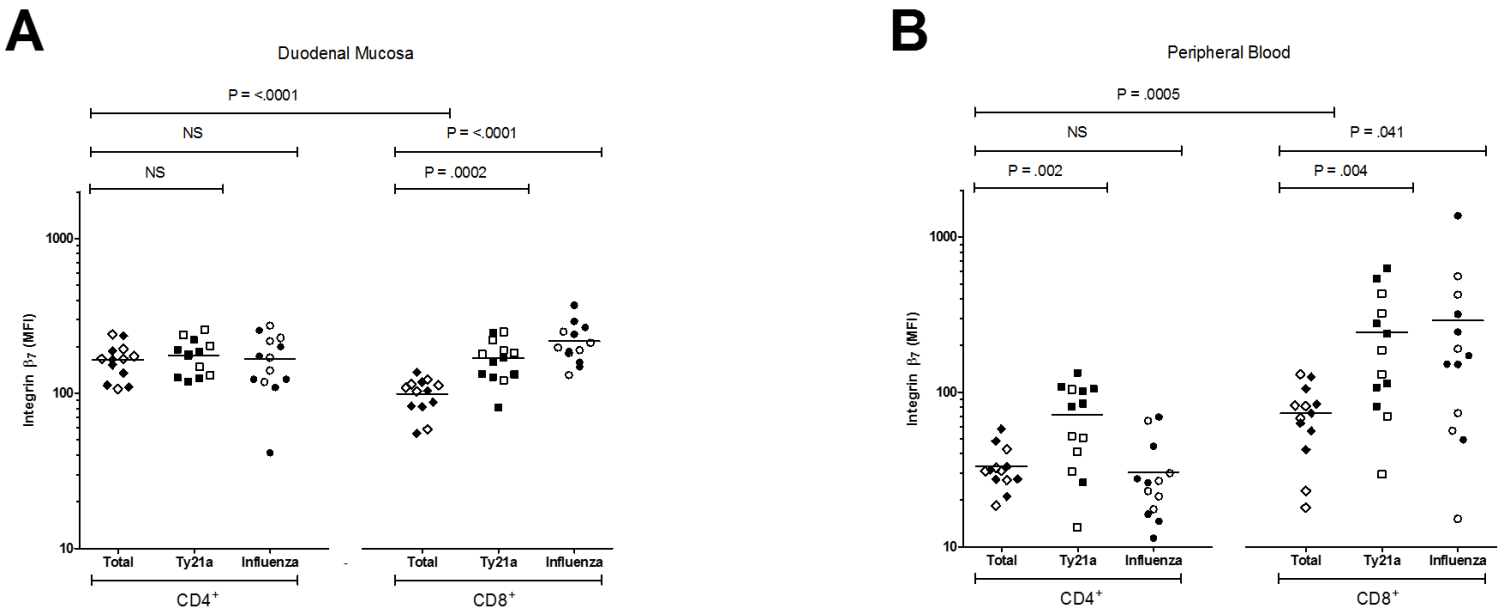

Figure 3.7. Integrin β_7 expression intensity among T-cell populations at day 18

Geometric mean fluorescence intensity (MFI) expression of integrin β_7 among non-stimulated total $CD4^+$ and total $CD8^+$ T-cell populations (control volunteers, closed diamonds [duodenal mucosa; n = 7] [peripheral blood; n = 7]; vaccinated volunteers, open diamonds [duodenal mucosa; n = 6] [peripheral blood; n = 6]) and in live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a)-stimulated (control volunteers, closed squares; vaccinated volunteers [duodenal mucosa; n = 7] [peripheral blood; n = 7], open squares [duodenal mucosa; n = 6] [peripheral blood; n = 6]) and heterologous influenza virus-stimulated (control volunteers, closed circles [duodenal mucosa; n = 7] [peripheral blood; n = 7]; vaccinated volunteers, open circles [duodenal mucosa; n = 6] [peripheral blood; n = 6]) cytokine-producing subpopulations. Measurements were made at the duodenal mucosa (A) and in peripheral blood (B). Horizontal bars represent mean values (comparisons were made using unpaired *t* tests). Abbreviation: ns, not significant.

3.4. Discussion

We have described, for the first time, the cellular response to oral vaccination with Ty21a at the human intestinal mucosa. We demonstrate that Ty21a vaccination generates both polyfunctional Ty21a-responsive and heterologous influenza virus-responsive T cells at the duodenal mucosa. The frequency of Ty21a-responsive and influenza virus-responsive T cells at the duodenal mucosa were robustly and consistently correlated. In contrast, cellular and humoral measurements made in peripheral blood were poorly correlated with one another and did not provide insight into the induction of heterologous responses at the duodenal mucosa.

While robust Ty21a and heterologous influenza virus-responsive $CD4^+$ and $CD8^+$ T cells were observed at the duodenal mucosa, no cellular response was observed at the colonic mucosa to either antigen in either T-cell subset. This suggests that duodenal responses were compartmentalised to the embryological midgut, which includes the terminal ileum, the likely site of active invasion by Ty21a (Parry et al., 2002). Interestingly, the frequency of Ty21a-responsive $CD4^+$ and $CD8^+$ T cells correlated robustly with one another and with the frequency of heterologous influenza virus-responsive $CD4^+$ and $CD8^+$ T cells at the duodenal mucosa. This suggests that the mechanisms responsible for the generation of these mucosal responses were localised and capable of influencing T cells of different phenotypes and specificities.

Polyfunctionality among Ty21a-responsive T cells at the duodenal mucosa and in peripheral blood may point toward an important mechanism through which Ty21a confers protection against disease (Darrah et al., 2007, Kannanganat et al., 2007). Further, the generation of anti-LPS immunoglobulin, an indication of vaccine efficacy, was closely associated with cellular responses at the duodenal mucosa, suggesting that mucosal cellular activity may be associated with the generation of protective immunity.

In murine models, influenza virus infection results in the recruitment of previously primed heterologous T cells to the lung (Chapman et al., 2005). We propose that a similar mechanism was responsible for the generation of heterologous responses at the duodenal mucosa. Although only 2 individuals had previously been vaccinated against influenza, all were likely to have been exposed to this mucosal pathogen in the community – indeed, at baseline, anti-influenza virus IgG and IgA, as well as influenza virus-responsive $CD4^+$ T cells, were detected in all volunteers, not just those who had been previously vaccinated against influenza.

Trafficking to the mucosa is largely dependent upon the expression of $\alpha_4\beta_7$ and its ligand, MAdCAM-1, as well as CCR9 and its ligand, CCL25 (Mavigner et al., 2012). We hypothesize that vaccination with Ty21a upregulated the expression of mucosal homing ligands resulting in the nonspecific migration of previously primed heterologous influenza virus-responsive T cells, carrying mucosal homing receptors, to the duodenal mucosa. This effect may or may not be restricted to influenza virus-responsive T-cell subsets and further study is warranted.

We observed that integrin β_7 expression intensity was generally higher among both Ty21a-responsive and influenza virus-responsive cells, compared with total cell populations, in peripheral blood and at the intestinal mucosa. Multiphasic peripheral $CD8^+$ T-cell responses have previously been described and attributed to the trafficking of immune cells from peripheral blood to the mucosa (Salerno-Goncalves et al., 2010, McArthur and Sztein, 2012). In keeping with this, integrin β_7 expression among peripheral $CD8^+$ T cells, particularly antigen-specific subpopulations, was more intense than that among peripheral $CD4^+$ T cells. Integrin β_7 expression among $CD4^+$ T-cell subsets was highly polarised, being relatively low among peripheral subsets and high among duodenal subsets. Differential integrin β_7 expression among antigen-specific $CD4^+$ T-cell populations may ensure that some peripheral T cells persist in blood and avoid sequestration. We and others have demonstrated the presence of intracellular intravascular and bone-marrow bacteria in early, late and recurrent

invasive *Salmonella* disease (Wain et al., 1998, Gordon et al., 2010). Thus, while T cells trafficking to the mucosa likely play a key role in the early response to invasion, $CD4^+$ T-cell populations that persist in peripheral blood may help to prevent intravascular dissemination and persistence at secondary systemic sites of infection.

Prior to vaccination, all volunteers had detectable baseline levels of serum immunoglobulin specific to *S.* Typhi LPS. This may be caused by immunoglobulin binding to the core or a result of environmental exposure to non-typhoidal strains bearing the same LPS O-antigens as *S.* Typhi (O-9 and O-12); *S. enterica* serovar Enteritidis expresses both O-9 and O-12 antigens and *S. enterica* serovar Typhimurium expresses the O-12 antigen (Kantele et al., 2012). Not all vaccinated volunteers generated peripheral humoral anti-LPS responses, which is likely a reflection of the limited efficacy of this vaccine (Anwar et al., 2014). It was interesting that mucosal cellular responses correlated strongly with serum anti-LPS immunoglobulin responses, as humoral responses to LPS have been associated with vaccine efficacy (Levine et al., 1989). This suggests that, in individuals in whom Ty21a vaccination is not efficacious, the failure encompasses both cellular and humoral mechanisms of defence. It has previously been demonstrated that the generation of immunoglobulin following oral vaccination is dependent upon $\alpha_4\beta_7$ (Wyant et al., 2015). Thus, the close association between cellular and humoral immune responses suggests that the strength of each volunteer's cellular and humoral response to vaccination was influenced by a mucosal mechanism, possibly the expression intensity of $\alpha_4\beta_7$ and/or MAdCAM-1 at the time of vaccination.

It is possible that, through TLR engagement, vaccination may have non-specifically activated mucosal antigen-presenting cells and T cells, resulting in enhanced cytokine production following experimental re-stimulation (Lore et al., 2003, Caron et al., 2005). However, as background cytokine production in the negative control was minimal and as cytokine production in the positive control was not different in the vaccinated volunteers as compared

to the unvaccinated volunteers, we believe this is unlikely to be the primary factor responsible for the observed heterologous cellular response. Owing to the invasive nature of endoscopic biopsy, we were limited to sampling from intestinal sites at a single time point. Data presented here and elsewhere (McArthur and Sztein, 2012) indicate that the assessment of mucosal immunity at alternate time points could provide further insight into the generation of protective immune responses. We assessed the expression of just 3 cytokines and, as a result, the T-cell populations identified here are unlikely to represent the responsive populations in their entirety. Variation within the human population may mean that we are underpowered to observe some other important biological effects.

3.5. Conclusion

Taken together, our data demonstrate that oral vaccination with Ty21a generates Ty21a-responsive T cells as well as heterologous influenza virus-responsive T cells at the duodenal mucosa. We propose that heterologous influenza virus-responsive T cells previously primed through natural exposure were recruited to the duodenal mucosa through compartmentalised upregulation of homing ligands. The direct evaluation of mucosal cellular immune defence provides the opportunity to identify functional correlates of protection and may offer new insights into mechanisms that may be manipulated to improve oral vaccine immunogenicity, either through the development of oral adjuvants or the development of multivalent *Salmonella* based vectors.

CHAPTER 4: THE ADAPTIVE IMMUNE RESPONSE TO TY21A AT APPROXIMATELY 1.5 YEARS

4.1. Introduction

Ty21a is able to induce humoral and cellular immune responses, both of which have been implicated in protection against disease. The peripheral humoral response to Ty21a has not previously been assessed beyond 42 days (Lundgren et al., 2009); however, one novel live-attenuated oral vaccine candidate, CVD 909, has demonstrated the capacity to generate memory B cells which persist for at least 1 year (Wahid et al., 2011). Peripheral cellular responses targeting soluble *S.* Typhi flagella (FliC) as well as infected host cells have been assessed following vaccination with Ty21a, and data indicate that T cells can persists for at least 2 years post-vaccination (Salerno-Goncalves et al., 2002, Salerno-Goncalves et al., 2010, McArthur and Sztein, 2012). Recently, it has been demonstrated that polyfunctional $CD8^+$ T cells are associated with protection against typhoid fever (Fresnay et al., 2016). We have previously demonstrated that vaccination with Ty21a generates robust $CD4^+$ and $CD8^+$ T-cell

responses at the duodenal mucosa at day 18 (Pennington et al., 2016); however, whether early duodenal responses persist in the long-term has yet to be determined.

An increased understanding of the longevity of immune responses both at the intestinal mucosa and in peripheral blood may allow us to identify functional correlates of vaccine-mediated protection, which are currently unknown. Here, we have assessed cellular immunity in vaccinated volunteers at the duodenal mucosa and in peripheral blood after approximately 1.5 years and compared responses with those observed in a control group. We have compared and correlated peripheral and mucosal cellular responses with peripheral levels of anti-LPS IgG and IgA, an indication of vaccine efficacy, providing a unique insight into the longevity of human mucosal and peripheral immune defence.

4.1.1. Hypotheses

We hypothesised that:

- Responses to Ty21a at the intestinal mucosa and in peripheral blood would persist

4.2. Methods

Full details of the methods used to produce the data which are presented within this chapter can be found in Chapter 2. Some data which are pertinent to this chapter are included for ease of reference.

4.2.1. Ethical approval and recruitment

Full details can be found in section 2.1. The following is a table of volunteer demographics:

Table 4.1. Chapter 4 volunteer demographics

Group	*n*	Gender (M:F)
Control	10	5:5
Vaccine	8	3:5

4.2.2. Vaccination

Full details can be found in section 2.3.

4.2.3. Clinical sampling

Full details of venesection and endoscopy can be found in sections 2.4.2 and 2.4.3, respectively.

4.2.4. Serum isolation

Full details can be found in section 2.5.4.

4.2.5. ELISA

Full details of ELISAs specific for LPS can be found in section 2.5.12.

4.2.6. PBMC isolation

Full details can be found in section 2.5.5.

4.2.7. MMC isolation

Full details can be found in section 2.5.7.

4.2.8. Ex vivo cell stimulation and staining

Full details of the antigens used for stimulation can be found in section 2.5.9. Antigens used in the chapter include:

Table 4.2. Antigens used for *in vitro* stimulation

Description	Preparation of stimuli
Negative control	100 µL complete medium
Heat-killed Ty21a	100 µL complete medium containing 1×10^6 CFU heat-killed Ty21a
Flagella (FliC)	100 µL complete medium containing 5 µg FliC protein
SEB	100 µL complete medium containing 100 ng SEB

Full details of the stimulation and staining protocol can be found in section 2.5.10. Details of the dyes and antibodies used in this chapter:

Table 4.3. Dyes and antibodies used for analysis of intracellular cytokine production

	Target	Clone	Fluorophore	Manufacturer	Per test
Viability	Dead cells	N/A	Pacific Blue	LifeTechnologies	0.02 µL
Extracellular	CD3	OCT3	BV/510	BioLegend	1 µL
	CD4	SK3	PE/Cy7	BD Biosciences	1 µL
	CD8	SK1	APC/H7	BD Biosciences	1 µL
	CD14	MϕP9	HorizonV450	BD Biosciences	1 µL
	CD19	HIB19	HorizonV450	BD Biosciences	1 µL
Intracellular	CD69	FN50	PE/CF594	BD Biosciences	2 µL
	MIP-1β	24006	APC	R&D Systems	2 µL
	IFN-γ	B27	AF700	BD Biosciences	2 µL
	TNF-α	MAb11	AF488	BD Biosciences	2 µL
	IL-2	MQ1-17H12	PE	BD Biosciences	2 µL
	IL-17A	eBio64DEC17	PerCP/Cy5.5	eBioscience	2 µL

4.2.9. Flow cytometric acquisition and analyses

Full details can be found in section 2.5.11. A BD LSR II was used for the acquisition of data.

4.2.10. Data management and statistical analysis

Full details can be found in section 2.6.

4.3. Results

4.3.1. Recruitment and sampling

Volunteers who had previously been vaccinated as part of past studies were recalled for sampling. The period between vaccination and sampling varied, with the median period between vaccination and sampling at 1.5 years (Table 4.4).

Table 4.4. Volunteer vaccination and sampling information

Study Number	Time from Vaccination to Sampling		
	Days	Years	Median (Years)
4539/02	599	1.6	1.5
4539/03	515	1.4	
4539/05	522	1.4	
4539/07	412	1.1	
4539/09	550	1.5	
4539/12	585	1.6	
4539/13	550	1.5	
4539/14	1,350	3.7	

4.3.2. Serum immunoglobulin specificity

Ty21a-mediated protection is dependent upon the expression of LPS (Germanier and Fuer, 1975) and, in field trials, humoral responses to LPS were shown to correlate with vaccine efficacy (Levine et al., 1989). We measured levels of serum anti-LPS IgG and IgA in vaccinated volunteers and controls.

At day 0 (baseline), levels of anti-LPS IgG and IgA did not differ between vaccinated and unvaccinated volunteers (Figure 4.1). Among vaccinated volunteers, levels of anti-LPS serum IgG were 6-fold higher at day 11 (T1), 5-fold higher at day 18 (T2) and 2-fold higher at approximately 1.5 years (T3) ([bootstrapped 95% confidence interval (CI) based on arbitrary units (AU)]: -54874 to -7404, -44577 to -5922 and -8911 to -317, respectively; Figure 4.1) than at baseline (T0).

Similarly, among vaccinated volunteers, levels of anti-LPS serum IgA were 3-fold higher among vaccinated volunteers at day 11 and 2-fold at day 18 (-97104 to -27173 and -41746 to -12843, respectively; Figure 4.1) than at baseline. Levels of anti-LPS serum IgA at approximately 1.5 years were comparable with baseline.

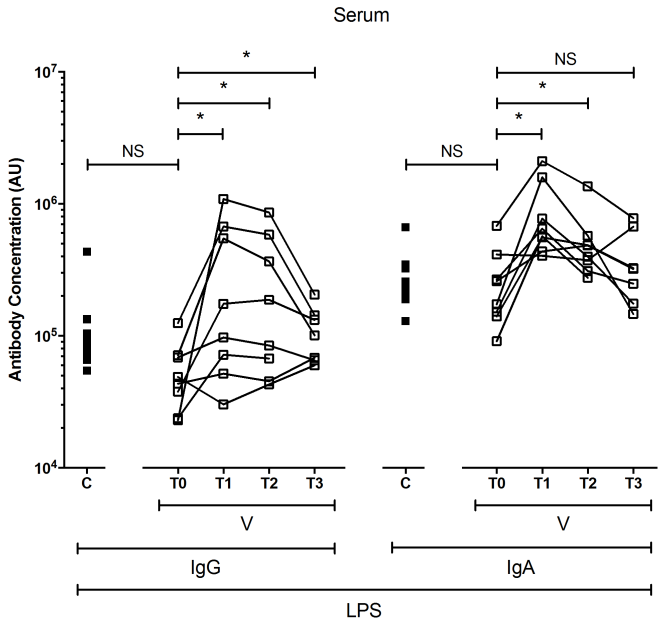

Figure 4.1. Levels of serum immunoglobulin (Ig)G and IgA to *Salmonella* Typhi lipopolysaccharide (LPS)

The levels of IgG and IgA specific to *Salmonella* Typhi LPS in serum, expressed in arbitrary units (AU). Unpaired comparisons were made between control (C; closed squares [n = 9]) and vaccinated (V; open squares [T0 n = 8; T1 n = 8; T2 n = 8; T3 n = 7]) volunteers at baseline (unpaired *t* tests were performed using logarithmically transformed data). Paired comparisons were made between baseline (T0) and day 11 (T1), day 18 (T2) and approximately 1.5 years (T3) (paired *t* tests were performed using logarithmically transformed data). Abbreviation: ns, not significant; *, bootstrapped 95% confidence interval does not cross 0.

4.3.3. Peripheral blood and gut mucosal cellular responses

We compared the frequency of Ty21a-responsive and FliC-responsive T cells in vaccinated volunteers and controls, at the duodenal mucosa and in peripheral blood. A combinatorial gating strategy was used to identify antigen-responsive cell populations; these were defined as the proportion of $CD4^+$ and $CD8^+$ T cells positive for any combination of IFN-γ ± TNF-α ± IL-2 ± IL-17A ± MIP-1β following re-stimulation (Figure 4.2). Cytokine production in non-

stimulated samples (negative control) was minimal, did not differ between vaccinated and unvaccinated volunteers and was subsequently subtracted from other conditions. Cytokine production in SEB-stimulated samples (positive control) was high and did not differ between vaccinated and unvaccinated volunteers.

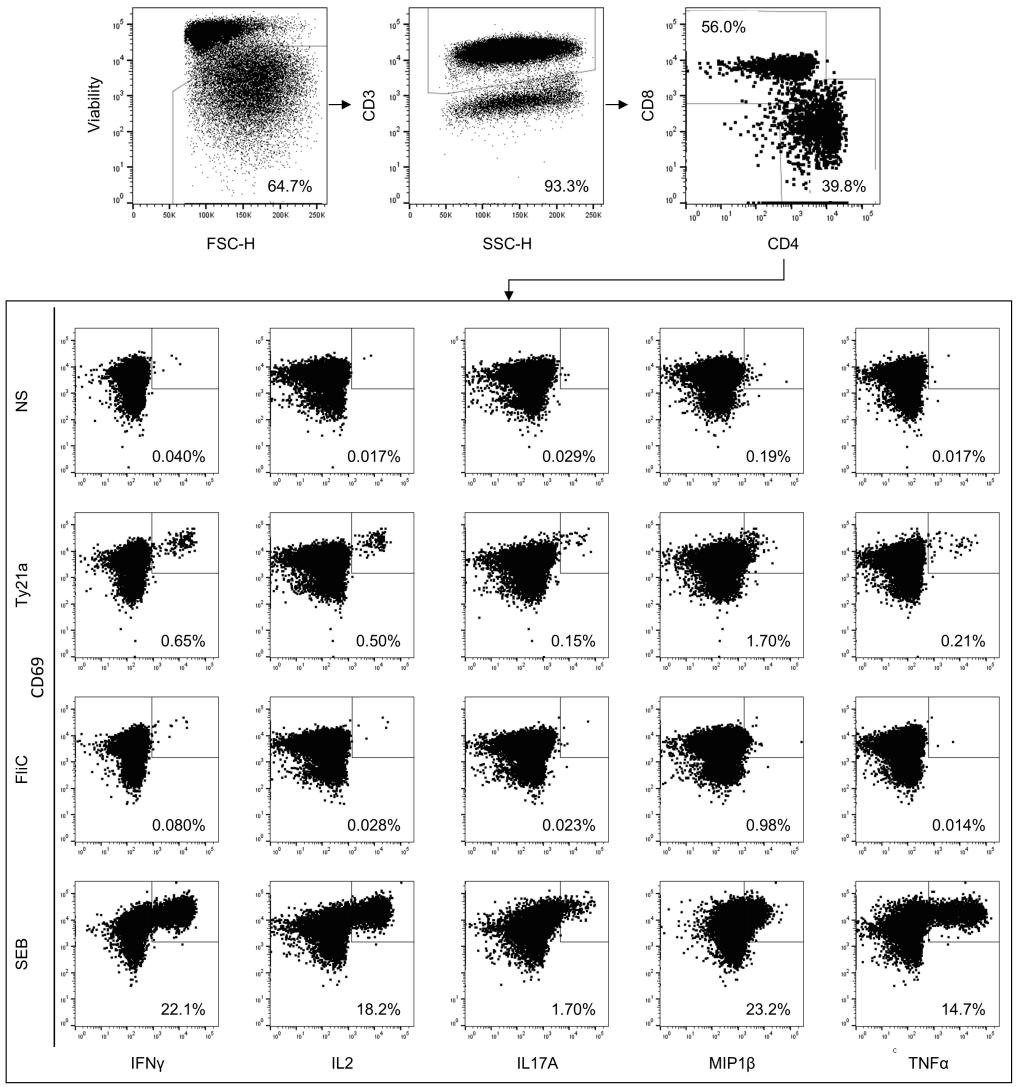

Figure 4.2 (A). Representative flow cytometric gating strategy for intracellular cytokine analysis

Dot plots are shown for cells isolated from the duodenal mucosa. Dead cells were removed by staining for viability (LIVE/DEAD) and gating on the negative population. T cells were identified according to the expression of CD3. T cells were classified according to the expression of CD4 and CD8 and the expression of IFN-γ, TNF-α, IL2, IL-17A, and MIP-1β assessed in non-stimulated (NS) and in Ty21a, FliC and SEB stimulated samples.

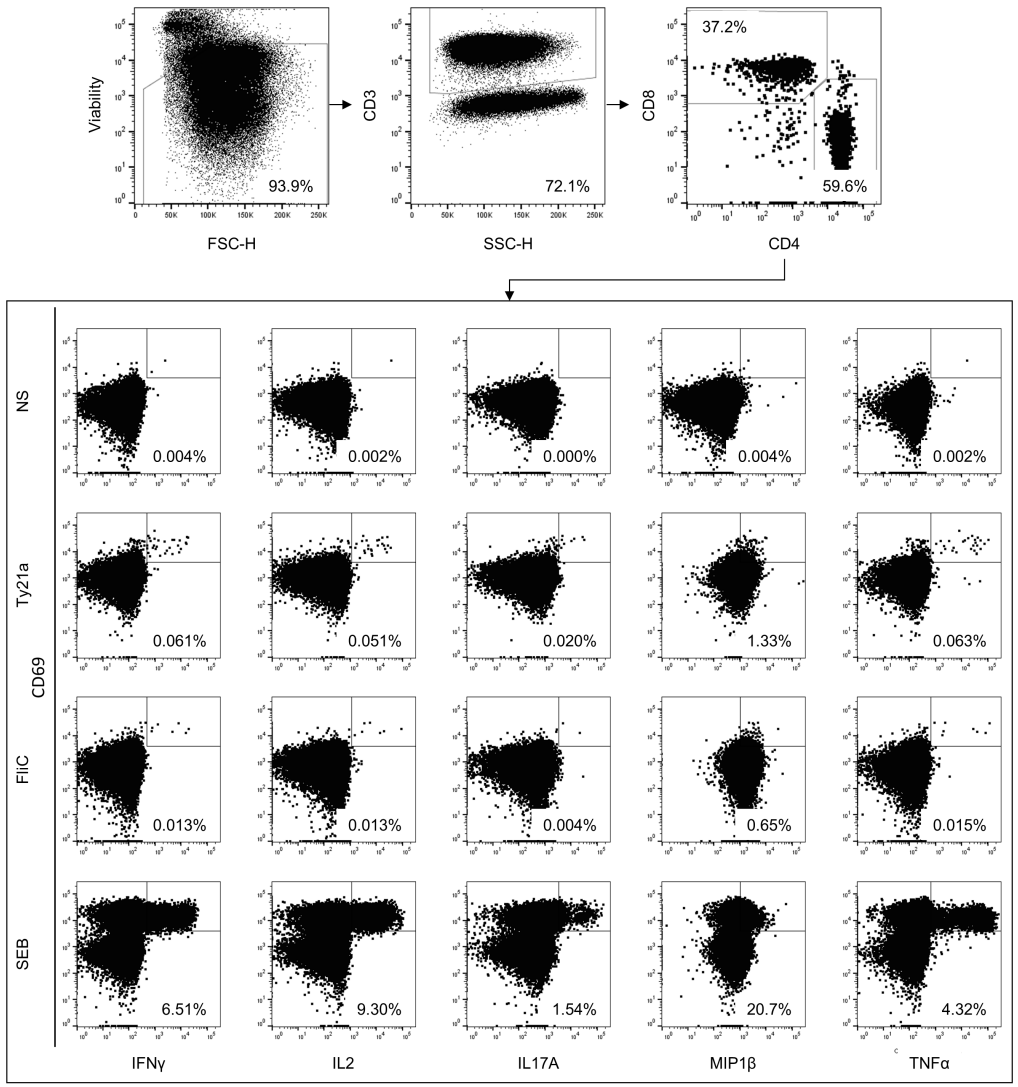

Figure 4.2 (B). Representative flow cytometric gating strategy for intracellular cytokine analysis

Dot plots are shown for cells isolated from peripheral blood. Dead cells were removed by staining for viability (LIVE/DEAD) and gating on the negative population. T cells were identified according to the expression of CD3. T cells were classified according to the expression of CD4 and CD8 and the expression of IFN-γ, TNF-α, IL2, IL-17A, and MIP-1β assessed in non-stimulated (NS) and in Ty21a, FliC and SEB stimulated samples.

Approximately 1.5 years following vaccination, at the duodenal mucosa, the frequencies of Ty21a-responsive and FliC-responsive $CD4^+$ and $CD8^+$ T cells in the vaccinated group was not different from the unvaccinated control group (Figure 4.3A).

Since overnight fasting, required prior to endoscopy, is known to influence cytokine production in peripheral blood in response to re-stimulation with bacterial antigens (van den Brink et al., 2002), we acquired non-fasting peripheral blood samples 6 days prior to gastroscopy. In peripheral blood, the frequency of Ty21a-responsive $CD4^+$ and $CD8^+$ T cells was not significantly higher in the vaccinated group compared to the unvaccinated control group (Figure 4.3B). The frequency of FliC-responsive $CD4^+$ T cells was 6-fold higher and $CD8^+$ T cells 2-fold higher in the vaccinated group, compared with the unvaccinated control group (([bootstrapped 95% CI based on percentage positive for combination of cytokine]: -3.48161 to -0.05863 and -5.46152 to -0.22464, respectively; Figure 4.3B).

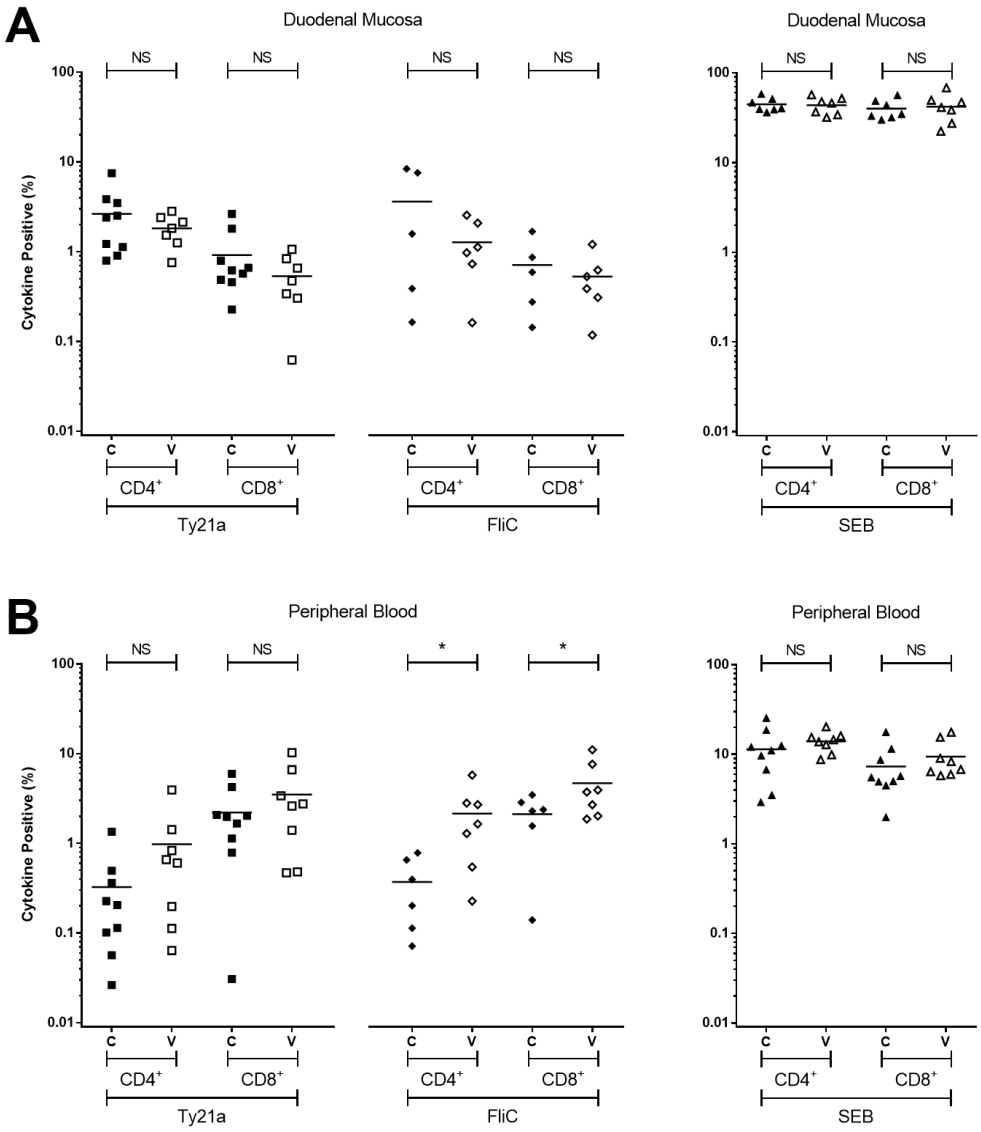

Figure 4.3. Antigen-specific cytokine-producing populations at 1.5 years

The frequency of $CD4^+$ and $CD8^+$ *Salmonella* Typhi strain Ty21a-responsive and FliC-responsive populations expressing any combination of IFN-γ ± TNF-α ± IL-2 ± IL-17A ± MIP-1β above background. SEB-stimulated control data are also included. For control (C; closed squares, diamonds and triangles [duodenal mucosa; Ty21a n = 9, FliC n = 5, SEB n = 7] [peripheral blood; Ty21a n = 9, FliC n = 6, SEB n = 9]) and vaccinated (V; open squares, diamonds and triangles [duodenal mucosa; Ty21a n = 7, FliC n = 6, SEB n = 7] [peripheral blood; Ty21a n = 8, FliC n = 7, SEB n = 8]) volunteers, measurements were made at the duodenal mucosa (A), and in peripheral blood (B). Horizontal bars represent mean values (comparisons were made using unpaired *t* tests).

4.3.4. Characteristics and functionality of cellular responses

Polyfunctional T cells, defined as cells that express multiple cytokines/chemokines simultaneously, have been shown to correlate with vaccine-mediated protection against other intracellular infections (Darrah et al., 2007, Kannanganat et al., 2007). After comparing the proportions of antigen-responsive populations, we assessed the cytokine expression profile of vaccinated volunteers with that of unvaccinated volunteers. Specifically, we assessed the functionality of the response as well as individual cytokine/chemokine production.

Consistent with our published data (Pennington et al., 2016), responses among $CD8^+$ T cells comprised far fewer polyfunctional subpopulations, both at the duodenal mucosa and in peripheral blood (Figure 4.4 and 4.6). Of the cytokines/chemokines studied here, MIP1β was consistently the most commonly expressed among Ty21a-responsive and FliC-responsive $CD4^+$ and $CD8^+$ T-cell populations, at the duodenal mucosa and in peripheral blood (Figure 4.5 and 4.7).

At the duodenal mucosa, the frequencies of Ty21a-responsive or FliC-responsive $CD4^+$ and $CD8^+$ T cells expressing one, two, three, four or five cytokines/chemokines in the vaccinated group was not different from the unvaccinated control group (Figure 4.4). Of the cytokines studied here, no discernible difference was observed between groups at the duodenal mucosa among either $CD4^+$ and $CD8^+$ T cells (Figure 4.5).

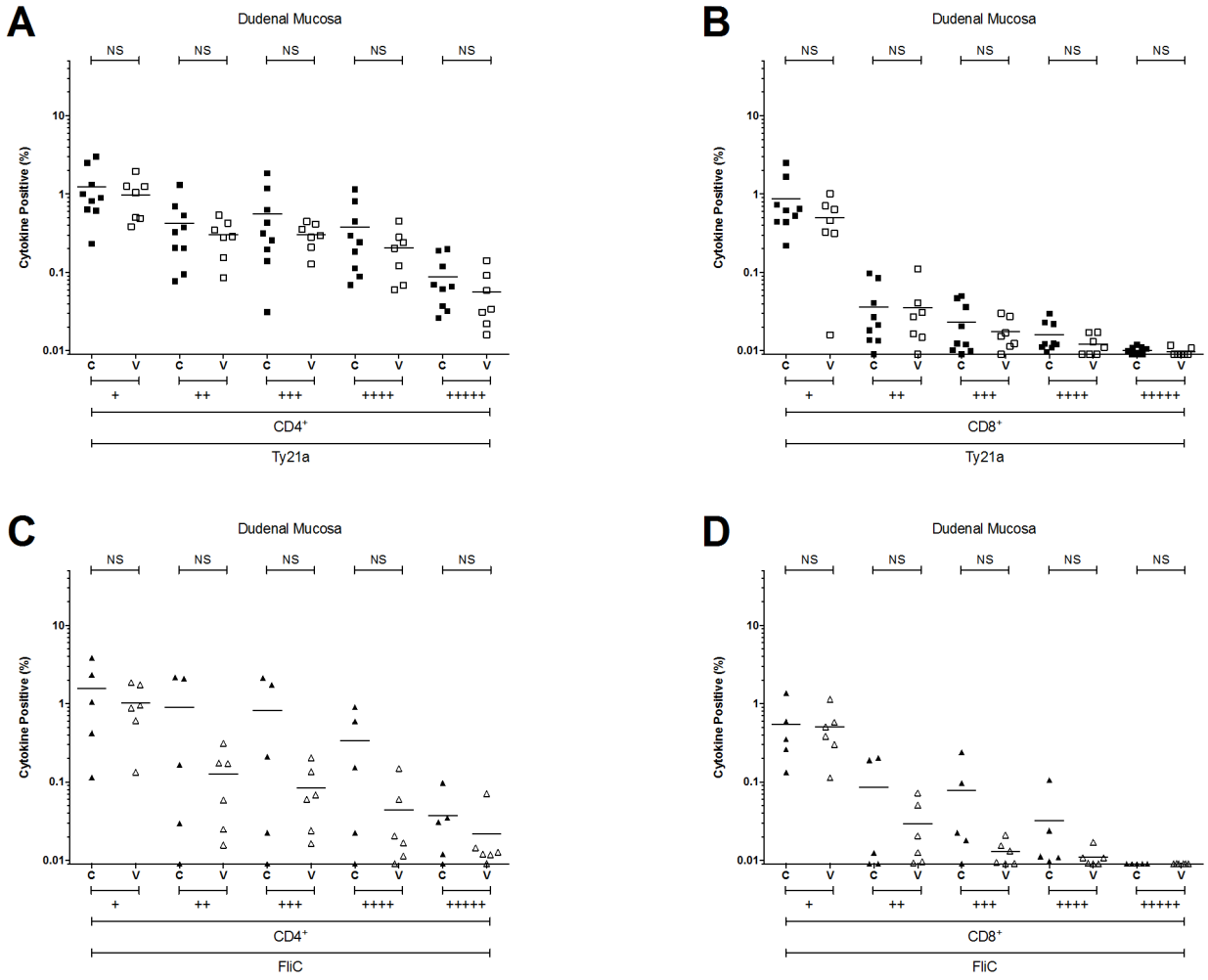

Figure 4.4. Combinations of antigen-specific cytokine production at the duodenal mucosa

The frequency of $CD4^+$ and $CD8^+$ *Salmonella* Typhi strain Ty21a-responsive (A and B) and FliC-responsive (C and D) populations expressing one (+), two (++), three (+++), four (++++) or five (+++++) cytokines/chemokines (IFN-γ ± TNF-α ± IL-2 ± IL-17A ± MIP-1β) above background. For control (C; closed squares and diamonds [Ty21a n = 9, FliC n = 5]) and vaccinated (V; open squares and diamonds [Ty21a n = 7, FliC n = 6]) volunteers. Horizontal bars represent mean values (comparisons were made using unpaired *t* tests).

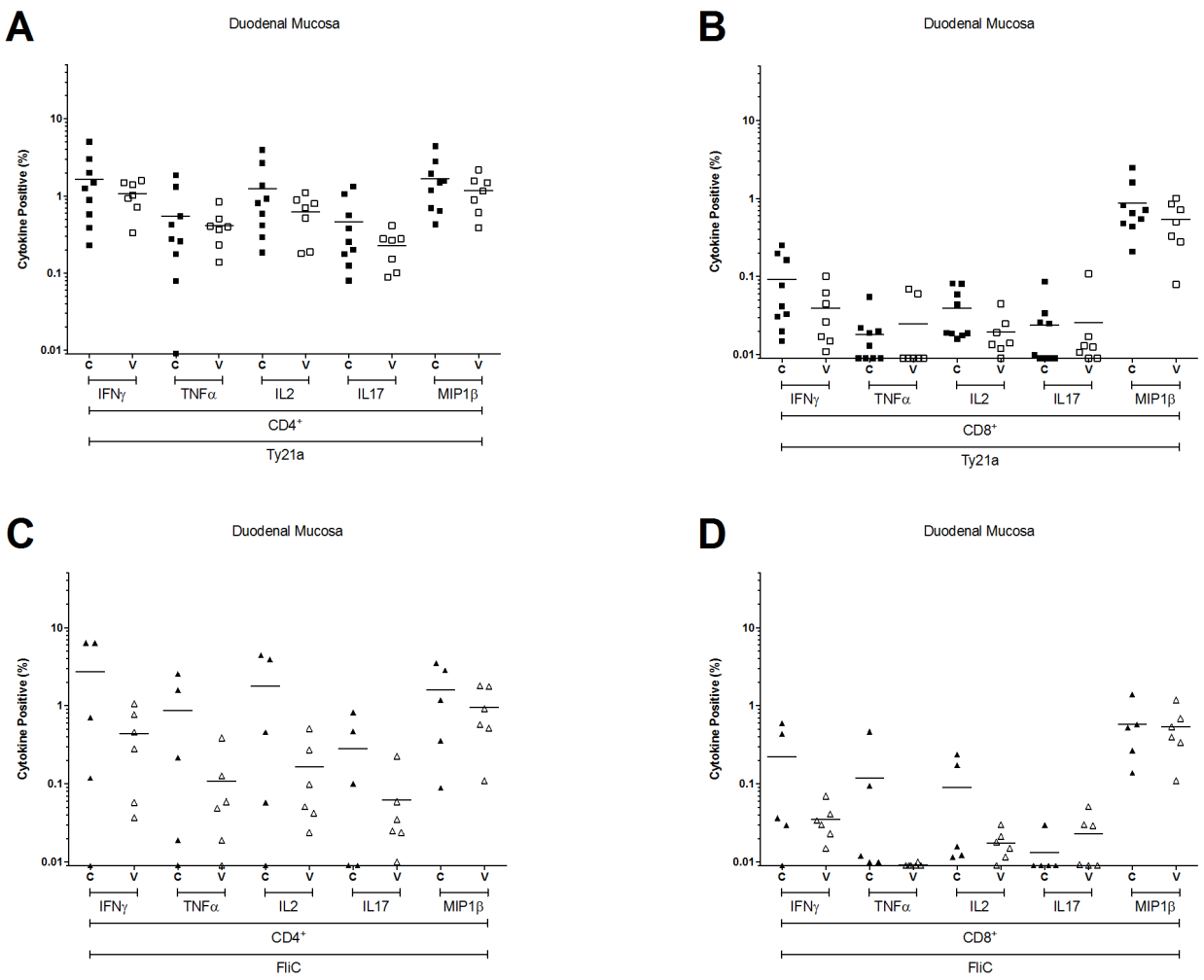

Figure 4.5. Antigen-specific production of individual cytokines at the duodenal mucosa

The frequency of $CD4^+$ and $CD8^+$ Ty21a-responsive (A and B) and FliC-responsive (C and D) populations expressing IFN-γ, TNF-α, IL-2, IL-17A, or MIP-1β above background. For control (C; closed squares and diamonds [Ty21a n = 9, FliC n = 5]) and vaccinated (V; open squares and diamonds [Ty21a n = 7, FliC n = 6]) volunteers. Horizontal bars represent mean values

In peripheral blood, the frequency of polyfunctional Ty21a-responsive $CD4^+$ T cells expressing four and five cytokines/chemokines was significantly higher in vaccinated volunteers than controls (-0.04385 to -0.00047 and -0.00810 to -0.00016, respectively; Figure 4.6). Consistent with the polyfunctional nature of these responses, the frequency of IFN-γ, TNF-α, IL-2, IL17 and MIP-1β expression among Ty21a-responsive $CD4^+$ T cells was increased (Figure 4.7). This suggests that the increased frequency of polyfunctional Ty21a-responsive $CD4^+$ T cells generated in response to vaccination persists for at least 1.5 years.

The frequency of FliC-responsive $CD4^+$ T cells expressing one and two cytokines/chemokines was higher in vaccinated volunteers than controls (-3.1896 to -0.52607 and -0.03688 to -0.00417, respectively; Figure 4.6). Analysis by individual cytokine revealed that the frequency of IFN-γ, TNF-α, IL-2, and MIP-1β expression among FliC-responsive $CD4^+$ T cells was increased (Figure 4.7).

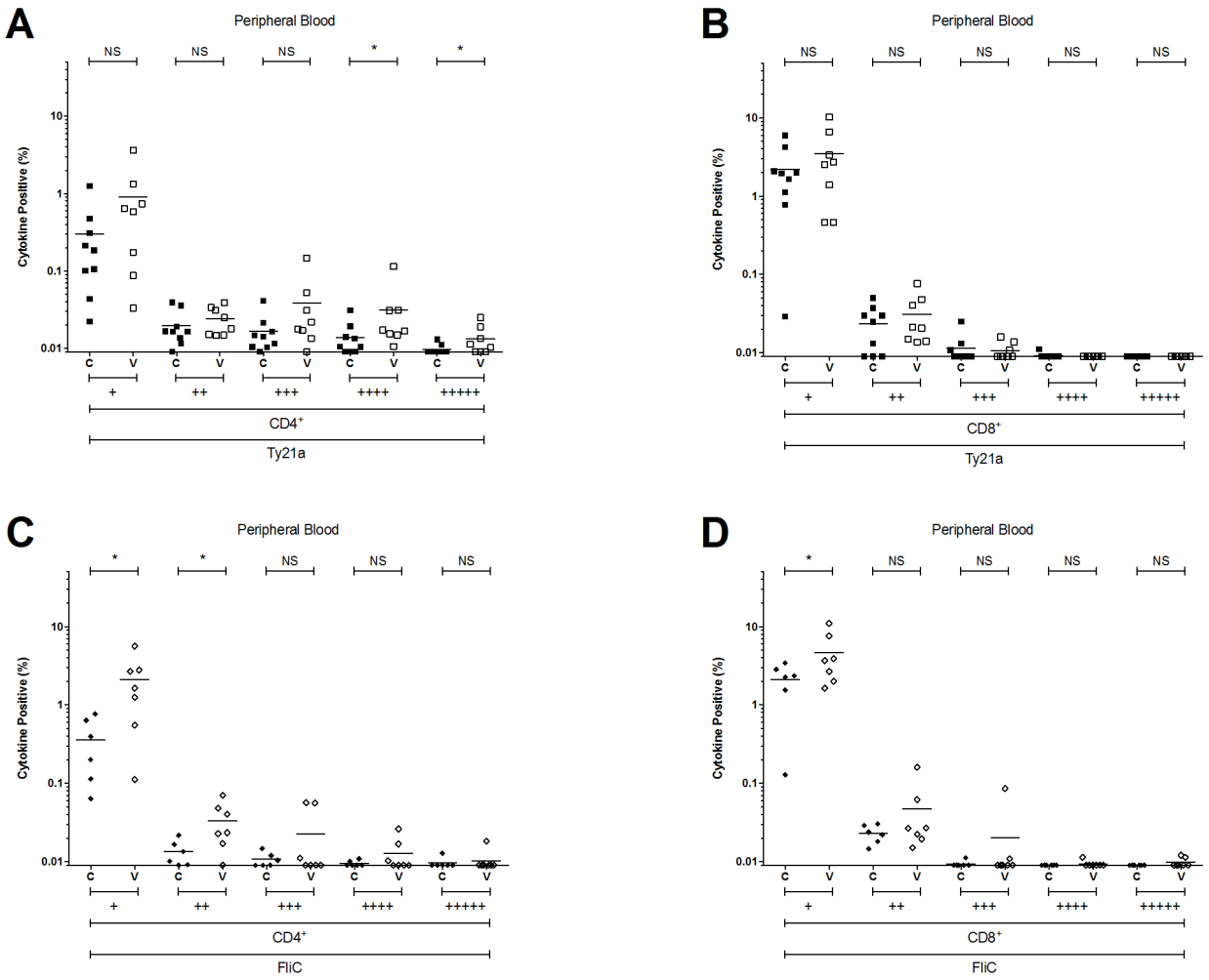

Figure 4.6. Combinations of antigen-specific cytokine production in peripheral blood

The frequency of $CD4^+$ and $CD8^+$ Ty21a-responsive (A and B) and FliC-responsive (C and D) populations expressing one (+), two (++), three (+++), four (++++) or five (+++++) cytokines/chemokines (IFN-γ ± TNF-α ± IL-2 ± IL1-7 ± MIP-1β) above background. For control (C; closed squares and diamonds [Ty21a n = 9, FliC n = 6]) and vaccinated (V; open squares and diamonds [Ty21a n = 8, FliC n = 7]) volunteers. Horizontal bars represent mean values (comparisons were made using unpaired *t* tests).

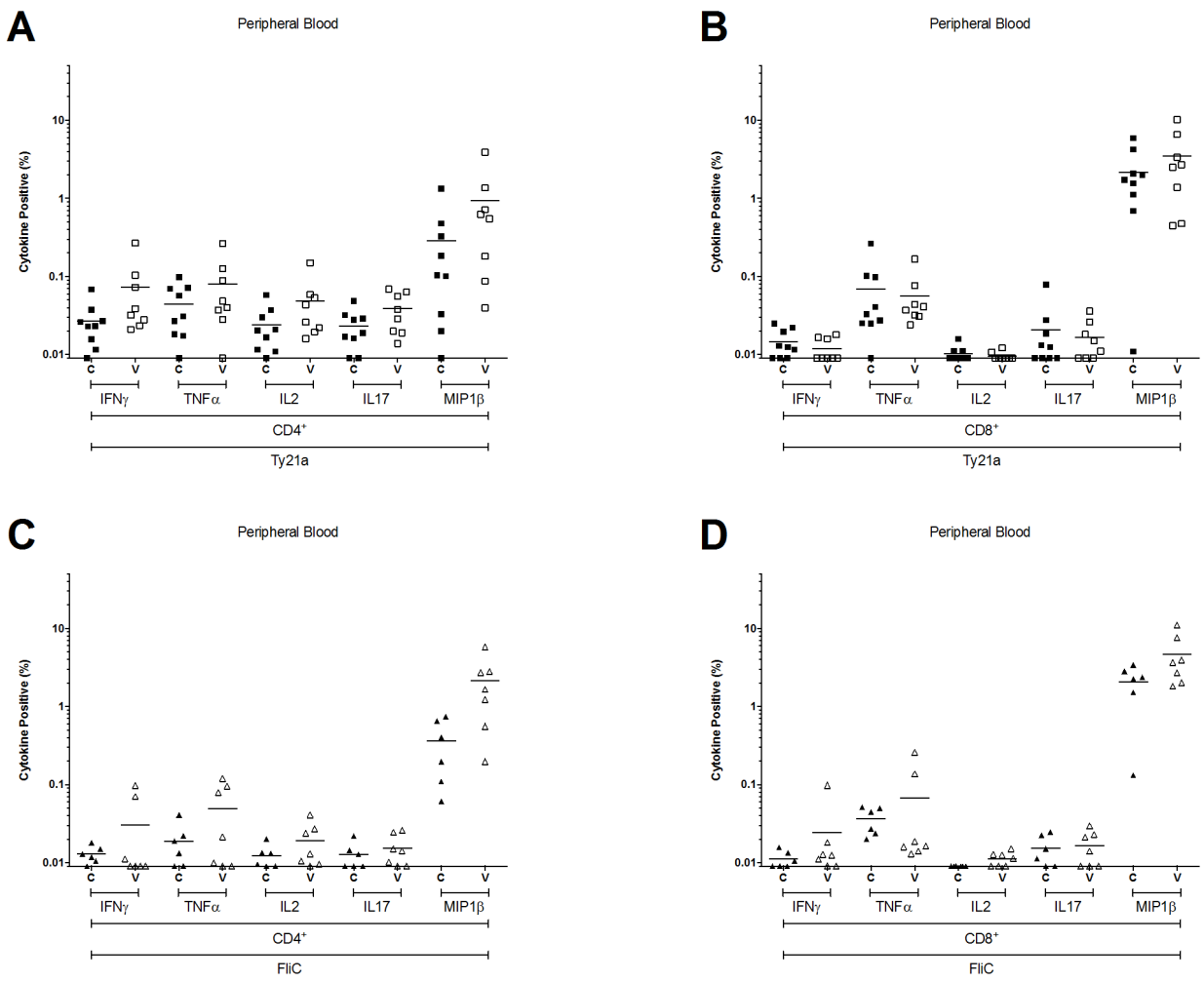

Figure 4.7. Antigen-specific production of individual cytokines in peripheral blood

The frequency of $CD4^+$ and $CD8^+$ Ty21a-responsive (A and B) and FliC-responsive (C and D) populations expressing IFN-γ, TNF-α, IL-2, IL-17A, or MIP-1β above background. For control (C; closed squares and diamonds [Ty21a n = 9, FliC n = 6]) and vaccinated (V; open squares and diamonds [Ty21a n = 8, FliC n = 7]) volunteers. Horizontal bars represent mean values.

4.3.5. Correlations between peripheral and mucosal immunity

We explored the relationship between peripheral humoral responses as well as the peripheral and duodenal cellular immune responses.

No correlation was observed between levels of anti-LPS serum IgG or IgA at any time point and the frequency of peripheral Ty21a-responsive or FliC-responsive $CD4^+$ or $CD8^+$ T cells at 1.5 years. Levels of anti-LPS serum IgA were, however, associated with the frequency of duodenal FliC-responsive $CD8^+$ T cells (data not shown). No other correlations were observed with duodenal T-cell populations.

4.4. Discussion

We have previously described the early mucosal response to live oral vaccination with Ty21a at the human duodenal mucosa, here we assessed the long-term cellular response to Ty21a at the same site and compared duodenal responses with peripheral responses. We demonstrate that, while peripheral polyfunctional cellular responses persist for at least 1.5 years, duodenal responses – which we have previously observed at day 18 (Pennington et al., 2016) – do not persist. The strength of early peripheral humoral responses to *S.* Typhi LPS, which have previously been associated with protective efficacy (Levine et al., 1989), were not associated with the strength of peripheral cellular responses at approximately 1.5 years.

We have previously reported the generation of Ty21a-responsive $CD4^+$ and $CD8^+$ T cells at the duodenal mucosa 18 days following vaccination with Ty21a. Here, approximately 1.5 years following vaccination, no response was observed at the duodenal mucosa in either T-cell subset. Interestingly, however, peripheral polyfunctional T-cell responses, generated through vaccination with Ty21a, do persist for at least 1.5 years. This suggests that duodenal responses are more transient than peripheral responses.

For some time the generation of polyfunctional T cells has been believed to be an important factor in conferring protection against typhoid fever (McArthur and Sztein, 2012, Maybeno et al., 2012, Fresnay et al., 2016, Wahid et al., 2016). Recently it has been demonstrated that, indeed, polyfunctional $CD8^+$ T cells are associated with protection against typhoid fever (Fresnay et al., 2016). Consistent with our previously published data (Pennington et al., 2016), the frequency of responsive cells tended to be higher amongst $CD4^+$ T cells; this is likely due to our use of soluble antigen preparations, which are dependent upon cross-presentation to engage cytotoxic $CD8^+$ T cells (Salerno-Goncalves et al., 2003, Wahid et al., 2008, Salerno-Goncalves and Sztein, 2009). Interestingly, while Ty21a-responsive $CD4^+$ T cells

comprised subsets which simultaneously expressed four and five cytokine/chemokines, FliC-responsive $CD4^+$ T cells tended to express just one or two cytokines/chemokines. This suggests that the FliC antigen is not responsible for the generation of polyfunctional responses and that other antigens, which are present in the heat-killed Ty21a preparation, are responsible for the induction of polyfunctional responses.

The fact that duodenal responses are transient, may indicate that the composition of mucosal T-cell populations is subject to considerable change. This may be due to the relatively high frequency with which different pathogens are encountered at the intestinal mucosa. It has previously been demonstrated that peripheral responses, generated through vaccination with Ty21a, with increased mucosal homing potential, persist in peripheral circulation for at least 90 days (Wahid et al., 2008). Thus, if *S*. Typhi were to be re-encountered at the intestinal mucosa, long-lived peripheral cell populations would likely possess an enhanced capacity to rapidly migrate to the mucosal surface through innate signalling and homing receptor up regulation (De Calisto et al., 2012).

Consistent with our previously published observations (Pennington et al., 2016), all volunteers had detectable baseline levels of serum immunoglobulin specific to *S.* Typhi LPS. This is likely the result of immunoglobulin binding to the core, or as a result of environmental exposure to non-typhoidal strains bearing the same LPS O-antigens as *S.* Typhi (O-9 and O-12) (Kantele et al., 2012). Not all vaccinated volunteers generated peripheral humoral anti-LPS responses; likely a reflection of the known limited efficacy of this vaccine (Anwar et al., 2014). While levels of serum anti-LPS IgG were significantly higher across all 3 time points, the strength of early responses steadily declined. Similarly, early anti-LPS IgA responses steadily declined, being significantly higher at day 11 and day 18, but returning to a level which was comparable with baseline at 1.5 years. In humans, memory B-cells can essentially persist for the life of the host (more than 50 years); however, typically, immunoglobulin

responses decline rapidly following clearance of the antigen (Schittek and Rajewsky, 1990, Hammarlund et al., 2003, Crotty et al., 2003). Thus, if the antigen is not reencountered *in vivo*, immunoglobulin responses are unlikely to be detected in the long-term.

The assessment of baseline immunoglobulin levels indicates that groups were well matched for prior exposure to *S.* Typhi. We have previously observed that heterologous influenza-responsive T-cell responses are generated at the duodenal mucosa 18 days following vaccination with Ty21a. Unfortunately, we were unable to determine the longevity of these responses in this study as the majority of the vaccinated cohort had been vaccinated against influenza in the period since being vaccinated with Ty21a (data not shown). We assessed the expression of four cytokines and one chemokine, as a result, the T-cell populations identified here are unlikely to represent the responsive populations in their entirety. Variation within the human population may mean that we are underpowered to observe some other important biological effects.

4.5. Conclusions

Taken together, our data demonstrate that early duodenal responses do not persist in the long-term. The fact that oral vaccination does generate peripheral populations which persist for at least 1.5 years supports the development of next-generation oral vaccines targeting typhoid, since it demonstrates longevity of orally induced cellular responses. Data presented elsewhere indicate that populations generated through oral vaccination express the homing molecules necessary to rapidly migrate to the intestinal mucosa following pathogenic exposure (Wahid et al., 2008). Controlled human infection, post-vaccination, may allow us to identify mechanisms responsible for efficacious defence against pathogens encountered at the intestinal mucosa and in the periphery.

CHAPTER 5: TRAINED IMMUNITY – NON-SPECIFIC EFFECTS OF ORAL VACCINATION WITH TY21A

5.1. Introduction

Epidemiological evidence has demonstrated that live-attenuated vaccines can reduce all-cause mortality (Benn et al., 2013, Goodridge et al., 2016). The strongest evidence has been collected in resource-poor settings following vaccination with BCG (Flanagan et al., 2013, Aaby et al., 2014). Similar effects have been observed following vaccination with measles containing vaccines and oral polio vaccines in both resource-poor and resource-rich settings (Aaby et al., 2010, Lund et al., 2015, Sorup et al., 2014, de Castro et al., 2015).

These non-specific beneficial effects are believed to be, in part, conferred through epigenetic modifications among innate immune cell populations (Ramirez-Carrozzi et al., 2006, Ramirez-Carrozzi et al., 2009, Krawczyk et al., 2010, Pantel et al., 2014). These changes may manifest themselves in the form of phenotypic variation among circulating innate cell

populations as well as altered cytokine production in response to *in vitro* cell stimulation (Kleinnijenhuis et al., 2014).

TLR-5 engagement at the mucosal surface of the gastrointestinal tract is known to enhance immune responses to influenza vaccination (Oh et al., 2014). It has been demonstrated that the live-attenuated oral typhoid vaccine, Ty21a, has the capacity to aid in the regression of bladder cancer, which is believed to be the result of TLR engagement (Domingos-Pereira et al., 2015). We have also observed enhanced cellular responses to influenza-virus at the duodenal mucosa 18 days following vaccination with Ty21a (Pennington et al., 2016). We, therefore, hypothesised that oral vaccination with Ty21a may possess the capacity to generate innate immune memory and alter responses to nonrelated pathogens.

The identification of bacteria able to induce trained immunity may lead to the development of adjuvants capable of enhancing vaccines targeting a wide array of pathogens. Here we have assessed the impact of vaccination with Ty21a on the expression intensity of several receptors expressed on the surface of on innate immune cells isolated from healthy volunteers at day 14, month 3 and month 6. In addition, we have assessed the impact of vaccination on IFN-γ (T_H1), IL-4 (T_H2), IL-17A (T_H17), TGF-β (T_{REG}) and TNF-α (T_H1) production among monocytes, TCRγδ, MAIT T cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD20^+$ B cells following *in vitro* stimulation.

5.1.1. Hypotheses

We hypothesised that:

- Vaccination with Ty21a would enhance the expression of surface receptors engaged by Ty21a on circulating $CD14^+$ monocytes
- Vaccination with Ty21a would enhance cytokine responses to nonrelated pathogens

5.2. Methods

Full details of the methods used to produce the data which are presented within this chapter can be found in Chapter 2. Some data which are pertinent to this chapter are included for ease of reference.

5.2.1. Ethical approval and recruitment

Full details can be found in section 2.1. The following is a table of volunteer demographics:

Table 5.1. Chapter 5 volunteer demographics

Group	*n*	Gender (M:F)
Control	13	2:11
Vaccine	17	4:13

5.2.2. Vaccination

Full details can be found in section 2.3.

5.2.3. Clinical sampling

Full details of venesection and endoscopy can be found in sections 2.4.2 and 2.4.3, respectively.

5.2.4. Serum isolation

Full details can be found in section 2.5.4.

5.2.5. ELISA

Full details of ELISAs specific for LPS and influenza-virus can be found in sections 2.5.12 and 2.5.13, respectively

5.2.6. PBMC isolation

Full details can be found in section 2.5.5.

5.2.1. PBMC thawing and overnight 'rest'

Full details can be found in section 2.5.6.

5.2.2. Phenotypic analyses of 'rested' monocytes

Full details can be found in section 2.5.8. Dyes and antibodies used for purposes of phenotypic analysis of monocytes in this chapter include:

Table 5.2. Dyes and antibodies used for analysis of monocyte phenotype

	Target	Clone	Fluorophore	Manufacturer	Per test
Viability	Dead cells	N/A	PacificOrange	LifeTechnologies	0.02 µL
Extracellular	CD14	FcγRIII	PerCP/Cy5.5	BD Biosciences	3.5 µL
	CD16	HI10a	APC/Cy7	BioLegend	3.5 µL
	CD64	TS1/18	BV605	BioLegend	3.5 µL
	CD18	ICRF44	PE/Cy7	BioLegend	3.5 µL
	CD11b	Bu15	AF700	BD Biosciences	3.5 µL
	CD11c	6H6	Pacific Blue	BioLegend	3.5 µL
	CD123	19.2	BV711	BioLegend	3.5 µL
	CD206	HTA125	PE/CF594	BD Biosciences	3.5 µL
	CD284	624915	APC	BioLegend	3.5 µL
	CD285	201A	FITC	R&D Systems	3.5 µL
	CD303	L243	PE	BioLegend	3.5 µL

5.2.3. Ex vivo cell stimulation and staining

Full details of the antigens used for stimulation can be found in section 2.5.9. Antigens used in the chapter include:

Table 5.3. Antigens used for *in vitro* stimulation

Description	Preparation of stimuli
Complete medium	100 µL complete medium
Heat-killed Ty21a	100 µL complete medium containing 2×10^6 CFU heat-killed Ty21a
Heat-killed *C. albicans*	100 µL complete medium containing 2×10^6 CFU heat-killed *C. albicans*
PPD from *M. tuberculosis*	100 µL complete medium containing 5 µg TB PPD
Tetanus toxoid	100 µL complete medium containing 20 µg tetanus toxoid
Split Viron Influenza	100 µL complete medium containing 180 ng hemagglutinin from, in equal quantities, a A/California/7/2009 H1N1-like strain, a A/Texas/50/20122 H3N2-like strain and a B/Massachusetts/2/2012-like strain

Full details of the stimulation and staining protocol can be found in section 2.5.10. Dyes and antibodies used for purposes of intracellular cytokine staining in this chapter include:

Table 5.4. Dyes and antibodies used for analysis of intracellular cytokine production

	Target	Clone	Fluorophore	Manufacturer	Per test
Viability	Dead cells	N/A	PacificOrange	LifeTechnologies	0.02 µL
Extracellular	CD3	SK7	APC/H7	BD Biosciences	1 µL
	CD4	SK3	PerCP/Cy5.5	BioLegend	1 µL
	CD8	SK1	BV/650	BioLegend	1 µL
	CD14	MϕP9	APC/H7	BD Biosciences	1 µL
	CD20	2H7	Pacific Blue	BioLegend	1 µL
	CD161	HP-3G10	BV/785	BioLegend	1 µL
	TCRγδ	11F2	PE/Cy7	BD Biosciences	1 µL
	Vα7.2	3C10	PE/Dazzle594	BioLegend	1 µL
Intracellular	IFN-γ	4S.B3	PE	BioLegend	2 µL
	TNF-α	MAb11	FITC	BioLegend	2 µL
	IL-17A	N49-653	AF700	BD Biosciences	2 µL
	IL-4	MP4-25D2	BV711	BD Biosciences	2 µL
	TGF-β	TW4-6H10	APC	BioLegend	2 µL

5.2.4. Flow cytometric acquisition and analyses

Full details can be found in section 2.5.11. A BD FACS Aria II was used for the acquisition of data.

5.2.5. Data management and statistical analysis

Full details can be found in section 2.6.

5.3. Results

5.3.1. Monocyte phenotype

We measured the relative expression intensity of CD11b (integrin α_M), CD11c (integrin α_X), CD14, CD16 (FcRIII), CD18 (integrin β_2), CD64 (FcγRI), CD123 (IL-3RA), CD206 (mannose receptor), CD303 (BDCA-2), HLA-DR, TLR-4 and TLR-5 on the surface of non-stimulated $CD14^+$ monocytes by flow cytometry. A sequential gating strategy was used to identify populations of interest (Figure 5.1). We compared the phenotypic properties of cells isolated from vaccinated volunteers and controls at day 0 with those isolated at day 14, month 3 and month 6.

We observed increased expression of CD11b, CD11c, CD16, CD64, CD303, TLR-4 and TLR-5 among vaccinated volunteers at day 14 (P = .002, P = .026, P = .005, P = .008, P = .027, P = .005 and P = .007, respectively; Figure 5.2). Increased expression of CD11b, CD16, CD303 and TLR-4 was observed at month 3 (P = .002, P = .042, P = .045 and P = .043, respectively; Figure 5.2). Expression of all receptors among vaccinated volunteers were comparable with baseline at month 6. No change in the expression intensity of CD14, CD18, CD123, CD206 and HLA-DR was observed among the vaccinated group at any time point. Among control group volunteers, increased expression of CD11b was observed at day 14 (P = .002). No change in the expression intensity of any other receptor at any time point was observed among the control group (Figure 5.3).

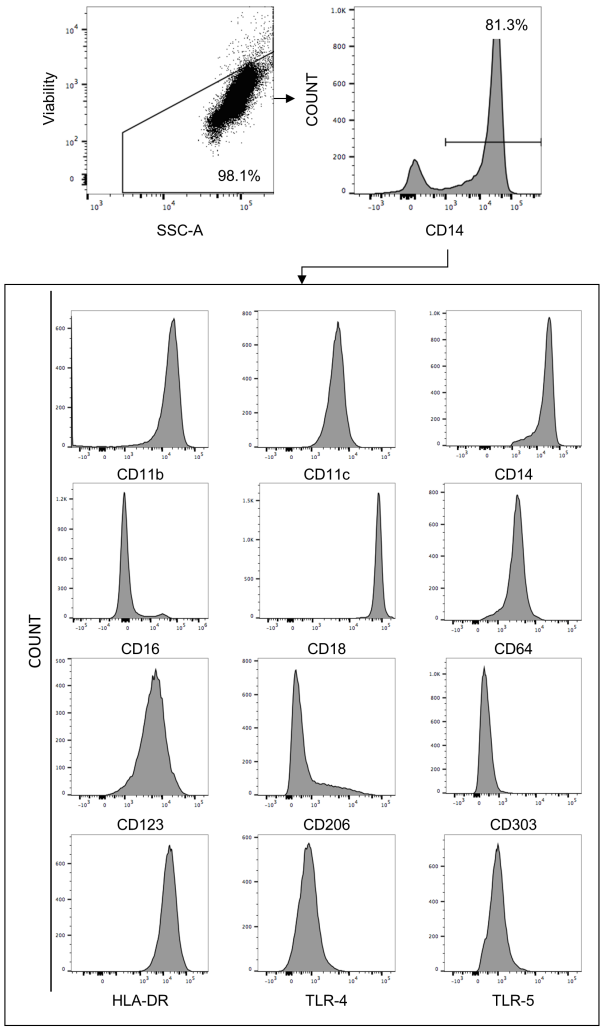

Figure 5.1. Representative flow cytometric gating strategy for phenotypic analysis of CD14$^+$ monocytes

Dot plots and histograms are shown for cells isolated from peripheral blood. Dead cells were removed by staining for viability (LIVE/DEAD) and gating on the negative population. Monocytes were identified according to the expression of CD14. The expression intensity of CD11b (integrin α_M), CD11c (integrin α_X), CD14, CD16 (FcRIII), CD18 (integrin β_2), CD64 (FcγRI), CD123 (IL-3RA), CD206 (mannose receptor), CD303 (BDCA-2), HLA-DR, TLR-4 and TLR-5 was assessed in non-stimulated samples.

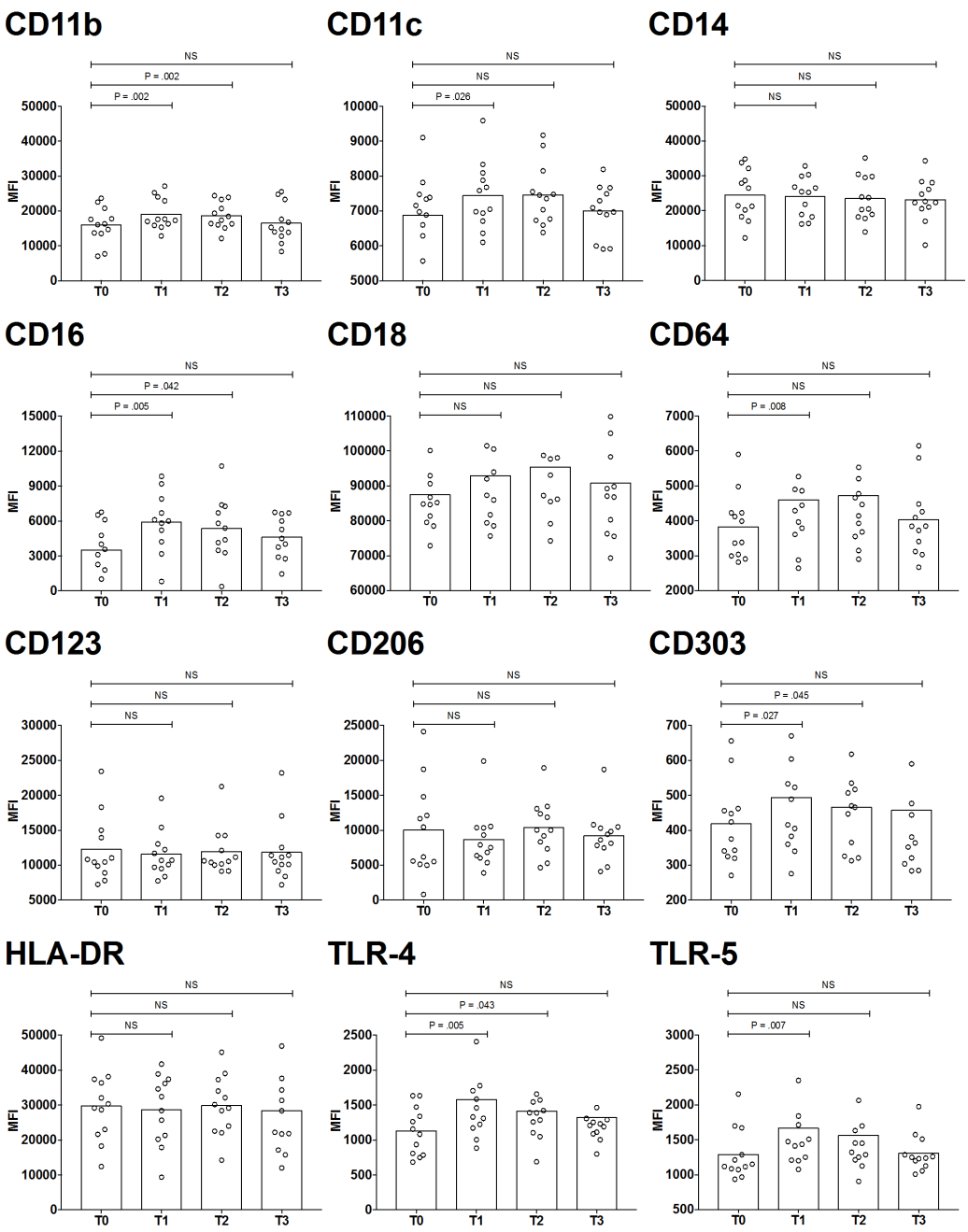

Figure 5.2. Surface receptor expression intensity among CD14$^+$ monocytes isolated from volunteers vaccinated with Ty21a

Geometric mean fluorescence intensity (MFI) expression of CD11b (integrin α_M), CD11c (integrin α_X), CD14, CD16 (FcRIII), CD18 (integrin β_2), CD64 (FcγRI), CD123 (IL-3RA), CD206 (mannose receptor), CD303 (BDCA-2), HLA-DR, TLR-4 and TLR-5 among CD14$^+$ monocytes isolated from volunteers vaccinated with *Salmonella* Typhi strain Ty21a (open circles). Samples were collected at day 0 (T0 n = 12), day 14 (T1 n = 12), month 3 (T2 n = 12) and month 6 (T3 n = 12). Bars represent mean values (comparisons were made using Wilcoxon matched-pairs signed rank tests). Abbreviation: ns, not significant.

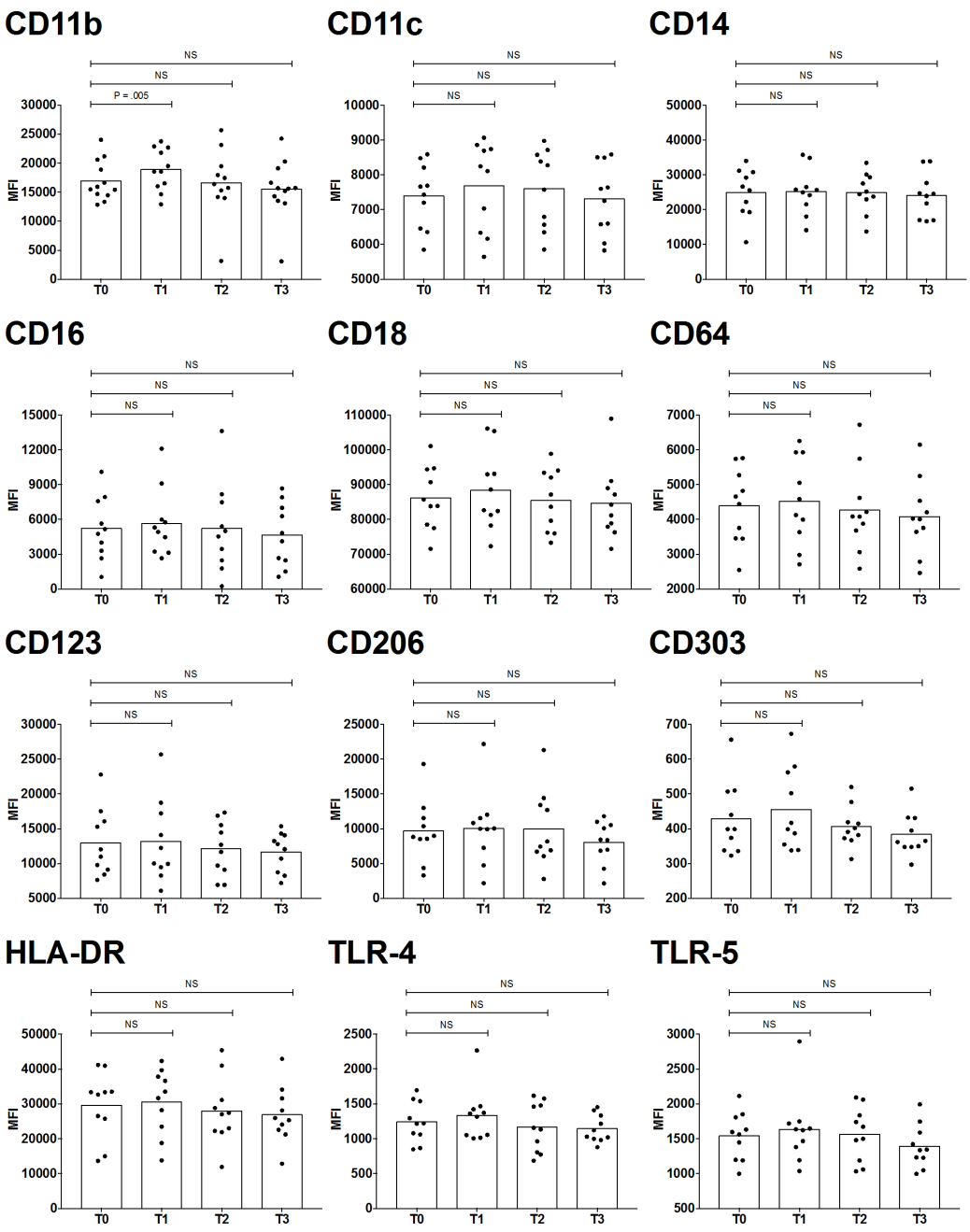

Figure 5.3. Surface receptor expression intensity among CD14$^+$ monocytes isolated from unvaccinated control group volunteers

Geometric mean fluorescence intensity (MFI) expression of CD11b (integrin α_M), CD11c (integrin α_X), CD14, CD16 (FcRIII), CD18 (integrin β_2), CD64 (FcγRI), CD123 (IL-3RA), CD206 (mannose receptor), CD303 (BDCA-2), HLA-DR, TLR-4 and TLR-5 among CD14$^+$ monocytes isolated from control group volunteers (closed circles). Samples were collected at day 0 (T0 n = 12), day 14 (T1 n = 11), month 3 (T2 n = 11) and month 6 (T3 n = 12). Bars represent mean values (comparisons were made using Wilcoxon matched-pairs signed rank tests). Abbreviation: ns, not significant.

5.3.2. Cytokine production

Cells were stimulated *in vitro* with a range of bacterial, fungal and viral antigens. Specifically, cells were stimulated with either heat-killed live-attenuated *Salmonella* Typhi strain Ty21a heat-killed *C. albicans*, split-viron influenza virus; PPD from *M. tuberculosis* and tetanus toxoid. We then assessed the production of IFN-γ, IL-4, IL-17A, TGF-β and TNF-α by B cells, $CD4^+$ T cells, $CD8^+$ T cells, monocytes, MAIT cells, and TCRγδ cells by flow cytometry. A sequential gating strategy was used to identify populations of interest (Figure 5.4). The frequency of each population positive for each cytokine was multiplied by the geometric mean fluorescence intensity for each cytokine-positive population above background – this well-established metric is known as the integrated mean fluorescence intensity (iMFI) (Darrah et al., 2007). Each volunteer was assigned a numerical value based on whether there was a reduction in output (-1; <80% of baseline), no change in output (0; between ≤80% and ≥120% of baseline), or increased output (+1; <120% of baseline) at T1, T2 and T3.

Temporal variation was observed in both the vaccinated group and the control group (Figure 5.5). Since we were not powered to perform comparisons where temporal variation was observed within the control group, we opted to model the data using DAPC to more generally assess whether vaccination had influenced heterologous *in vitro* cytokine production. When all stimuli were included for each cell type and cytokine, the model, comprising 30 principal components, reclassified all samples with an accuracy of 85.3% (Figure 5.6). Loadings data revealed the largest contributing cell populations were B cells, TCRγδ and $CD8^+$ T cells. Interestingly, when the Ty21a stimulus was removed, the accuracy of the model was virtually unchanged, and the model, comprising 30 principal components, reclassified all samples with an accuracy of 83.3% (Figure 5.7). Once more, loadings data revealed the largest contributing cell populations were B cells, TCRγδ and $CD8^+$ T cells.

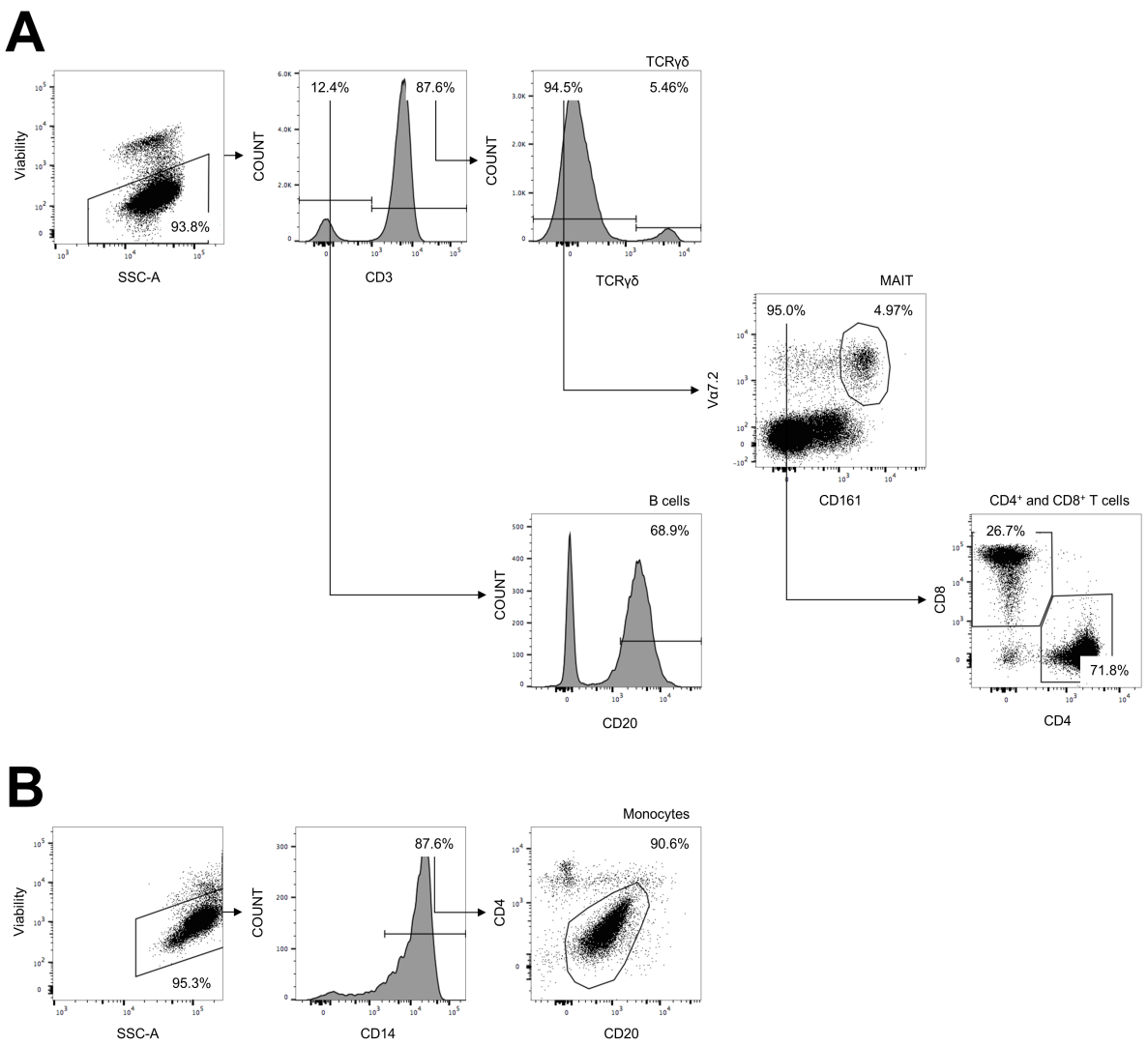

Figure 5.4. Representative flow cytometric gating strategy for intracellular cytokine analysis

Dot plots and histograms are shown for cells isolated from peripheral blood. (A) Dead cells were removed by staining for viability (LIVE/DEAD) and gating on the negative population. The CD3 positive population were defined as follows; TCRγδ cells were identified according to the expression of TCRγδ; mucosal associated invariant (MAIT) cells were identified according to the expression of CD161 and Vα7.2 not already identified as TCRγδ cells; $CD4^+$ and $CD8^+$ T cells were identified according to the expression of CD4 and CD8 not already identified as TCRγδ cells or MAIT cells. (B) Monocytes were identified according to the expression of CD14 as well as the intermediate expression of CD4 and CD20. The expression of interferon γ (IFN-γ), interleukin 4 (IL-4), IL-17A, transforming growth factor β (TGF-β), and/or tumor necrosis factor α (TNF-α) assessed in non-stimulated and in heat-killed live-attenuated *Salmonella* Typhi strain Ty21a; heat-killed Candida albicans; split-viron influenza; purified protein derivative from *Mycobacterium tuberculosis*; tetanus toxoid-stimulated samples.

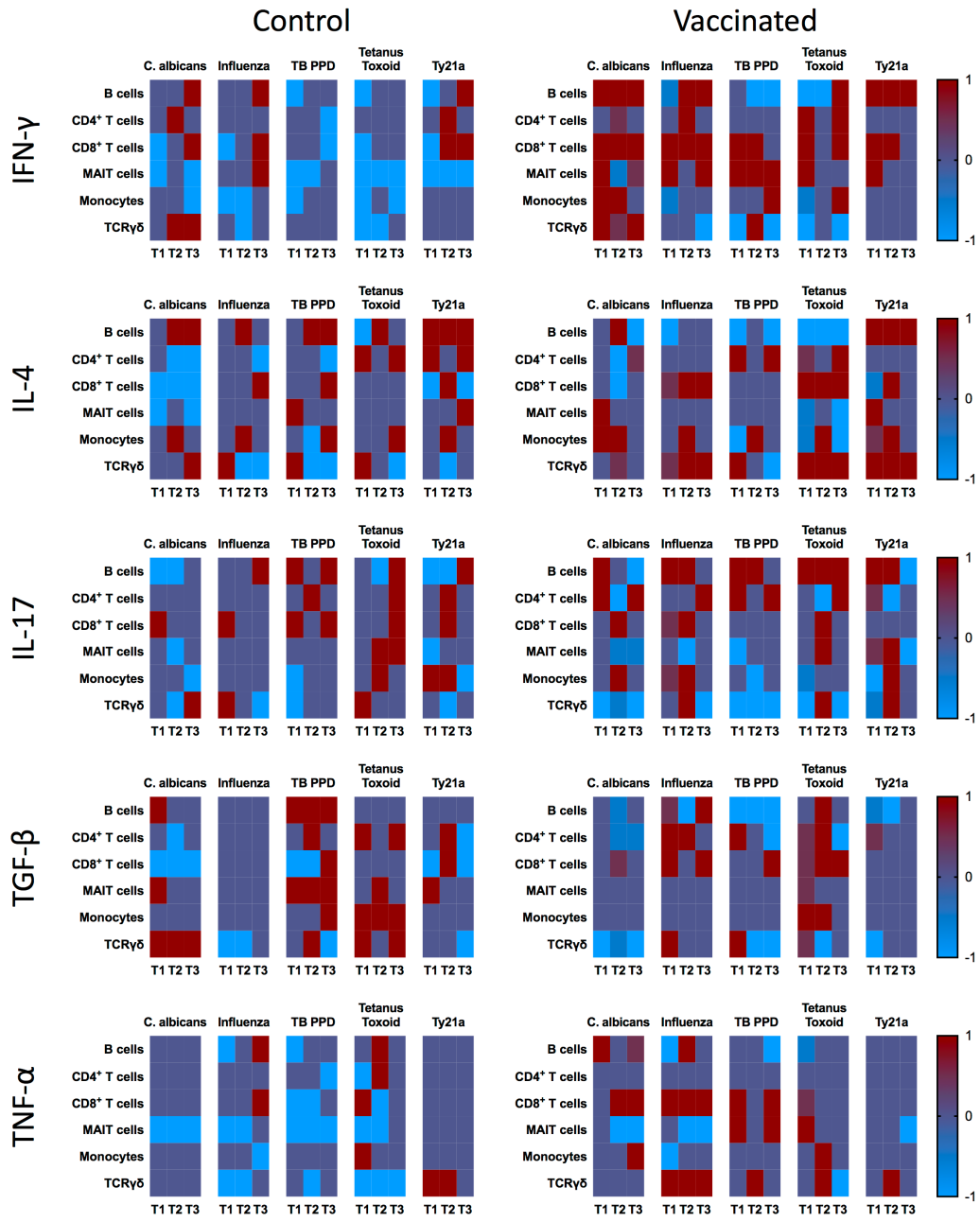

Figure 5.5. Heat map showing change in integrated mean fluorescence intensity (iMFI) amongst unvaccinated control group volunteers and volunteers vaccinated with *Salmonella* Typhi strain Ty21a

iMFI (IFN-γ was assessed at day 14 (T1), month 3 (T2), month 6 (T3) amongst B cells, $CD4^+$ T cells, $CD8^+$ T cells, monocytes, mucosal associated invariant (MAIT) cells, and TCRγδ cells and compared with baseline (T0). Data were compiled for each of the following stimuli; heat-killed live-attenuated *Salmonella* Typhi strain Ty21a (Ty21a); heat-killed *Candida albicans* (*C. albicans*); split-viron influenza (Influenza); purified protein derivative from *Mycobacterium tuberculosis* (TB PPD); tetanus toxoid. For each volunteer iMFI was calculated and each volunteer assigned a numerical value based on whether there was a reduction in output (-1; <80% of baseline), no change in output (0; between ≤80% and ≥120% of baseline), or increased output (+1; <120% of baseline) at T1, T2 and T3. The median for the group was then calculated and graphically represented.

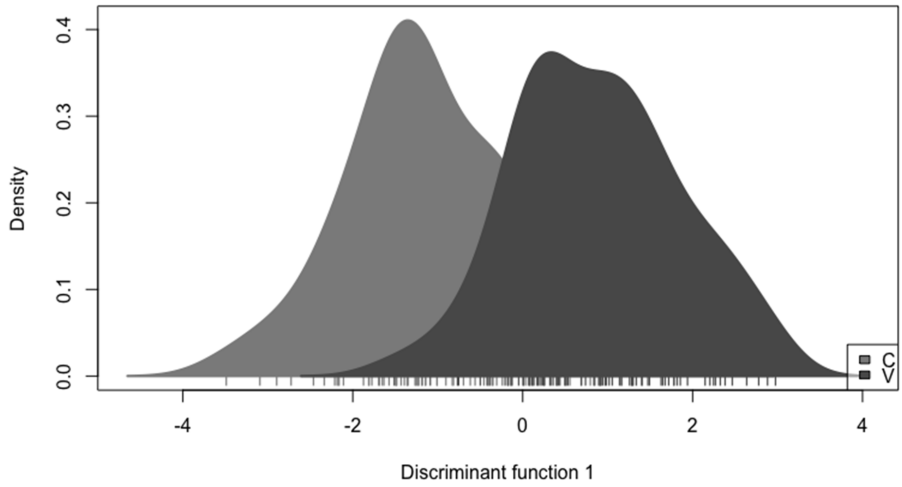

Figure 5.6. Density plots showing linear discriminant analysis of principal components (DAPC)

A model was built using data collected from control (C; light-grey) and vaccinated (V; mid-grey) volunteers. Data were compiled for the following stimuli; live-attenuated *Salmonella* Typhi strain Ty21a, *Candida albicans*, influenza virus, purified protein derivative from *Mycobacterium tuberculosis* and tetanus toxoid. Cytokine production (IFN-γ,at day 14 (T1), month 3 (T2) and month 6 (T3) amongst B cells, $CD4^+$ T cells, $CD8^+$ T cells, monocytes, mucosal associated invariant (MAIT) cells, and TCRγδ cells.

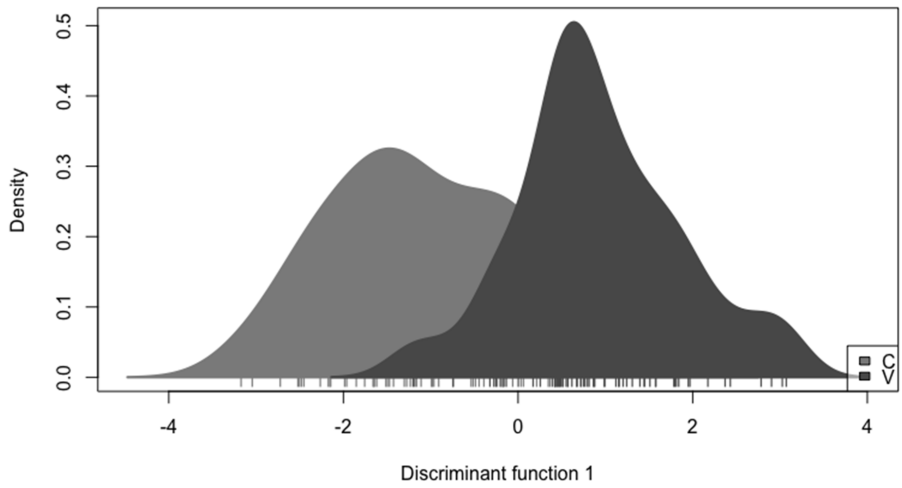

Figure 5.7. Density plots showing linear discriminant analysis of principal components (DAPC) using unrelated non-vaccine antigens only

A model was built using data collected from control (C; light-grey) and vaccinated (V; mid-grey) volunteers. Data were compiled for the following stimuli, *Candida albicans*, influenza virus, purified protein derivative from *Mycobacterium tuberculosis* and

tetanus toxoid, with the notable exclusion of the live-attenuated *Salmonella* Typhi strain Ty21a stimulus. Cytokine production (IFN-γat day 14 (T1), month 3 (T2) and month 6 (T3) amongst B cells, $CD4^+$ T cells, $CD8^+$ T cells, monocytes, mucosal associated invariant (MAIT) cells, and TCRγδ cells.

5.4. Discussion

Here we have demonstrated that oral vaccination with *S*. Typhi strain Ty21a can induce upregulation of CD11b, CD11c, CD16, CD64, CD303, TLR-4 and TLR-5 among $CD14^+$ monocytes for at least 3 months. These changes are indicative of the induction of trained immunity. We have further demonstrated that vaccination with Ty21a alters cytokine production among various cell populations in response to stimulation with non-related pathogens.

In studies assessing the generation of trained immunity, TLR4 as well as CD14, CD11b and CD206 has been shown to be upregulated among monocytes for at least 1 year following vaccination with BCG (Kleinnijenhuis et al., 2014). Challenge with wild-type *S*. Typhi has been shown to induce activation of circulating monocytes and DCs up to 96 hours following onset of disease (Toapanta et al., 2015). Here we have observed the increased expression of TLR-4 and TLR-5 on monocytes following vaccination with Ty21a; likely a response to the engagement of these TLRs by LPS and flagella, respectively. In addition to changes among TLRs, which are semi-pathogen-specific, we also observed the upregulation of CD11b, CD11c, CD16, CD64 and CD303, the upregulation of these molecules is associated with enhanced phagocytic function and antigen-presentation as well as enhanced chemotaxic potential (Solovjov et al., 2005, Georgakopoulos et al., 2008, Cheeseman et al., 2016). These changes likely contribute towards the non-specific beneficial effects conferred through the generation of trained immunity.

Differences in expression were observed for at least 3 months following vaccination. Since monocytes typically only persist in circulation for a single day (Yona et al., 2013), it is reasonable to hypothesise that phenotypic changes observed as long as 3 months after vaccination were the result of changes which occurred at the progenitor level. However, since no effect was observed at 6 months, it is likely that monocytes do eventually return to

a phenotype more in line with the individuals' natural immune state. Nevertheless, in the 3 months following vaccination, these changes are likely to have a significant impact on the capture and presentation of antigen by differentiated monocytes – DCs and macrophages – and the adaptive immune responses which are subsequently generated.

Many factors influence human cytokine production; indeed, variation has been reported over a period as short as 24 hours (Edgar et al., 2016). We have observed temporal variation in cytokine production in the unvaccinated control group over a 6 month period. It is possible that changes in the control group may be the result of seasonal variation in the relative frequency with which different pathogens are encountered. In order to ensure that effects, particularly those surrounding heterologous responses, are not wrongly attributed to vaccination or clinical intervention, it is imperative that future studies include temporally matched-controls in order to account for temporal variation, as was the case in this study. This is important since temporal variation may, at least in part, contribute toward altered cytokine production which has been observed elsewhere (Kleinnijenhuis et al., 2014).

BCG, measles containing vaccines and oral polio vaccines have all been associated with reductions in all-cause mortality (Aaby et al., 2010, Flanagan et al., 2013, Aaby et al., 2014, Sorup et al., 2014, Lund et al., 2015, de Castro et al., 2015); however, it is unclear whether these observations are the result of altered cytokine production. It has previously been demonstrated that cytokine production in response to *in vitro* stimulation with heterologous antigens is altered following vaccination with BCG (Kleinnijenhuis et al., 2014). It is unclear whether changes in cytokine production are the result of altered antigen-presentation occurring *in vitro*, or whether these changes reflect altered immune cell population composition occurring *in vivo.* To our knowledge, this is the first instance where the assessment of cytokine production by cell type has been attempted in the context of trained immunity, with previous studies having relied upon the assessment of supernatants collected

following *in vitro* stimulation (Kleinnijenhuis et al., 2014). The use of DAPC to interrogate our data has allowed us to make the general conclusion that vaccination with Ty21a does indeed alter cytokine production to non-related pathogens. Since, DAPC modelling revealed a similar capacity to reassign volunteers, even when Ty21a data were excluded, we can assume that the specific responses generated to the vaccine were not the only responses which enabled discrimination of the two groups. This suggests that vaccination with Ty21a did indeed alter cytokine responses to heterologous antigens. Unfortunately, since we did not anticipate temporal variation within the control group, we lacked the statistical power to perform the appropriate statistical comparisons to provide detailed insight into the populations driving this phenomenon. Variation within the human population may mean that we are underpowered to observe some other important biological effects. Evidence does, however, suggest a prominent role for B cells, TCRγδ and $CD8^+$ T cells in the altered responses which were observed. Further study is warranted to more fully characterise the role that innate, proinflamatory and regulatory cell types and effector molecules play in the generation of trained immunity.

5.5. Conclusion

Our data demonstrate that Ty21a possess the potential to induce changes associated with the generation of trained immunity. Although BCG, measles containing vaccines and oral polio vaccines have all been associated with the induction of trained immunity and a reduction in all-cause mortality (Aaby et al., 2010, Flanagan et al., 2013, Aaby et al., 2014, Sorup et al., 2014, Lund et al., 2015, de Castro et al., 2015), the low cost and ease with which Ty21a may be administered makes it a particularly attractive platform for development. However, to date, no data has been presented which demonstrates that these kind of immunological observations are clinically relevant and are responsible for a reduction in all-cause mortality in humans (Pollard et al., 2017). Data presented here would indicate that studies to determine whether vaccination with Ty21a also impacts all-cause mortality should be undertaken.

Further study is required to determine whether Ty21a may, be administered alongside other vaccines to enhance their efficacy. Since trained innate immune cells persist for at least 3 months following vaccination with Ty21a, other pathogens/antigens encountered during this period would be subject to altered recognition and presentation. As a result, the relatively short-lived impact of trained immunity – observed for just 3 months here – may well lead to lasting effects on subsequently generated adaptive immune responses, which can persist for the life of the host. These data support the development of *S.* Typhi as a vector to deliver unrelated antigens, since, through the induction of trained immunity, *S.* Typhi based vectors may confer additional benefits beyond those originally envisioned during the earliest stages of their development.

CHAPTER 6: GENERAL DISCUSSION

This thesis describes the assessment of the human immune response to oral vaccination with live-attenuated *S.* Typhi strain Ty21a in peripheral blood and at the intestinal mucosa. The three overarching objectives of this thesis were:

- To characterise the short-term impact of Ty21a on adaptive human immunity in peripheral blood and at the duodenal and colonic mucosa
- To characterise the long-term impact of Ty21a on adaptive human immunity in peripheral blood and at the duodenal mucosa
- To characterise the non-specific effects of Ty21a on innate cell phenotype and its impact on immune responses to an array of pathogens

6.1. Discussion of key findings

S. Typhi remains a considerable threat to global health. Although the efficacy of the Ty21a vaccine is limited (Anwar et al., 2014), a considerable amount of data have been collected concerning the peripheral cellular and humoral immune response to Ty21a, which has been

used to inform the development of next-generation vaccines. This thesis, however, explores areas previously unexplored, and is the first instance where the impact of Ty21a on cellular immunity at the human intestinal mucosa has been assessed, and where the induction of trained immunity has been associated with administration of this vaccine.

We have also demonstrated that Ty21a may be used as an immunological probe to study mucosal immune defence, and that Ty21a may be used to confer benefits beyond those specific to *S.* Typhi.

6.1.1. Peripheral cellular immune responses do not fully reflect cellular immune responses at the intestinal mucosa

Responses were observed at the duodenal mucosa which were stronger and more diverse than those observed in peripheral blood. The assessment of novel vaccines using peripheral sampling techniques alone may not fully reflect immunogenicity. Indeed, with regards orally-administered vaccines targeting mucosal pathogens, the direct assessment of mucosal immune defence is a more relevant measure of immunogenicity, which may more accurately reflect some aspects of oral vaccine efficacy. The mechanisms through which adaptive immune responses are generated during early exposure at the intestinal mucosa merits further study, including, but not limited to, the study of a wider range of cell types and the assessment of transcriptomic signals observed during early responses.

6.1.2. Intestinal cellular immune responses are compartmentalised

At the intestinal mucosa, cellular responses were observed at the duodenal mucosa which were not observed at the colonic mucosa. The total surface area of the adult human gastrointestinal mucosa is estimated to be between 30 and 40 m^2 (Helander and Fandriks, 2014); thus, compartmentalisation of immune cell trafficking likely ensures that cell populations accumulate in regions where they were initially primed and are, therefore, more likely to play an active role in immune defence.

6.1.3. Heterologous cellular immune responses are observed at the human intestinal mucosa

Cellular immune responses were observed to Ty21a as well as viral influenza antigens at the duodenal mucosa. We hypothesise that TLR engagement and cellular activity at the intestinal mucosa generates an innate response, which results in the migration of competent adaptive immune cells into the local space. This likely ensures that adaptive immune responses to any pathogen are generated as soon as possible following exposure.

6.1.4. Peripheral, but not intestinal, cellular responses persist in the long-term

Cellular immune responses were observed to persist in peripheral blood, but not at the duodenal mucosa approximately 1.5 years following vaccination. Due to the non-sterile nature of the intestinal mucosa, and the relative frequency with which pathogens are encountered at this site, the turnover of cells of various cell specificities is likely to be greater and more dynamic than in peripheral blood. Thus, immune responses generated in response to vaccination, at the intestinal mucosa, may be more difficult to detect in the long-term.

6.1.5. Trained immunity is conferred and immune responses to heterologous antigens are altered

Monocyte phenotype and cytokine expression profiles are altered following vaccination with Ty21a. Data presented here are the first which demonstrate that Ty21a generates innate immune memory. Phenotypic analysis of monocytes demonstrated increased expression of surface molecules engaged by Ty21a, as well as other associated which are indicative of a heightened immune state. Similar effects have previously been observed following vaccination with BCG (Kleinnijenhuis et al., 2014). The low cost and ease with which Ty21a may be administered, compared with other vaccines which have been shown to confer

innate immune memory, makes Ty21a a particularly attractive platform for further development (Aaby et al., 2010, Flanagan et al., 2013, Aaby et al., 2014, Sorup et al., 2014, Lund et al., 2015, de Castro et al., 2015). The administration of Ty21a alongside other vaccines could improve their efficacy. Further study is warranted to explore the impact of Ty21a on immune responses to co-administered vaccine antigens. Evidence would indicate that, for example, administration of Ty21a could enhance responses to parenterally-administered vaccines through TLR engagement (Oh et al., 2014).

6.2. Future work

Based on the data presented within this thesis, further investigation to more fully characterise the complex interactions between *S.* Typhi and its human host is warranted. Some possible avenues of exploration include:

6.2.1. The assessment of peripheral and mucosal immune cellular responses to wild-type *S.* Typhi

Our use of *S.* Typhi strain Ty21a has enabled us to study host-pathogen interaction with minimal risk to volunteers and at a fraction of the cost that would have been incurred if we were to study wild-type *S.* Typhi. Unfortunately, due to the nature of its generation, Ty21a contains a number of mutations, which makes it an imperfect model of true infection and host defence.

Since the first models of controlled human infection were discontinued, technological advancements have made it possible to safely acquire previously inaccessible samples. The acquisition and interrogation of these samples would allow for a more comprehensive assessment of host-pathogen interaction. The controlled *S.* Typhi human infection model now established at the University of Oxford, will utilise these advancements to study pathogenic wild-type *S.* Typhi in its natural host, both peripherally and at the intestinal

mucosa. These data will expand our understanding of disease pathogenesis and factors influencing susceptibility to infection.

6.2.1. Transcriptomic analysis of peripheral and mucosal responses *S.* Typhi strain Ty21a and wild-type *S.* Typhi

The assessment of the human transcriptome following exposure to Ty21a both at the intestinal mucosa and in peripheral blood could yield important insight into the mechanisms responsible for the generation of protective immune responses. Further, analysis of the transcriptome way provide insight into the mechanisms engaged which induce changes associated with the generation of trained immunity – this could lead to the development of refined molecules which could be administered alongside countless other vaccines as next-generation adjuvants.

The assessment of the human transcriptome following exposure to wild-type *S.* Typhi both at the intestinal mucosa and in peripheral blood would be of great interest. A greater understanding of factors which influence host susceptibility to disease could lead to the development of more effective vaccines targeting this pathogen. Transcriptomic analysis could also provide fresh insight into the mechanisms which *S.* Typhi utilises in order to circumvent human immune defence.

6.2.2. Combined delivery of Ty21a alongside other vaccines

Data presented here would suggest that combined administration of Ty21a alongside other vaccines may enhance their efficacy. Since intestinal TLR engagement is known to enhance the efficacy intramuscular vaccines (Oh et al., 2014), oral administration of Ty21a may possess the capacity to influence responses to a range of vaccines, not just those which are orally-administered.

A double-blind randomised control trial would be required to test this hypothesis. Recently, experimental human pneumococcal carriage has been used to successfully assess the efficacy of 13-valent pneumococcal conjugate vaccine (PCV) on pneumococcal colonisation, at a fraction of the cost associated with full-scale field trials (Collins et al., 2015). If, for example, the administration of Ty21a alongside PCV resulted in increased vaccine immunogenicity and improved efficacy, it would have important implications for the development of novel vaccines targeting the pneumococcus, as well as for the development of novel adjuvants, and for wider vaccine strategy.

6.3. Concluding remarks

We have demonstrated that Ty21a can be used to safely study cellular immune defence at the human intestinal mucosa. Thus, oral vaccination with Ty21a could be used as a model to study host-pathogen interaction at the human intestinal mucosa, and could provide insight into the generation of mucosal immune defence and insight into cellular dynamics at the intestinal mucosa. We have also demonstrated that Ty21a can confer additional benefits, beyond those specific to the targeted pathogen, influencing immune responses to non-related pathogens. These findings have enhanced our understanding of immune defence and have important implications for vaccine strategy and for the development of next-generation vaccine adjuvants.

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